SOME ASPECTS OF SEED BIOLOGY OF ROWAN

(*SORBUS AUCUPARIA* L.)

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DECLARATION

I hereby declare that this thesis has been composed entirely by me and that all the work herein was carried out by me alone, except where otherwise acknowledged.

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ABSTRACT

This study is in two parts. The first part is concerned with the study of dormancy, after-ripening and germination in *Sorbus aucuparia* L.(rowan) seeds. The second part deals with the development of a suitable micropropagation system from juvenile parts of the same species.

Seeds of rowan possess both testa-imposed and embryo dormancy at maturity.They have a wide range of depth of dormancy and germinated in the cold (4°C) after different periods of after-ripening. After-ripening of intact seeds at 1°C was found to be superior to that at 4°C by having an earlier Intensive Germination Period (IGP) and of shorter IGP duration. However, Total Germination % (TG%) was not significantly different. Freshly collected seeds were found to be the best in their TG % (90 %), with shortest duration and time arriving at IGP.

Alternating warm (20°C) period(2 weeks) with a cold (4°C) period (26 weeks) improved its TG % and IGP duration greatly but lengthened the time to IGP. TG and IGP duration of stored seeds could be improved by alternating warm and cold stratification.

The stratification medium can modify dormancy breaking during the cold stratification of seeds. The highest percentage of germination after 10 weeks of stratification (4°C) was obtained in the order of sand>vermiculite>peat>polythene bag. A mixture of sand and peat was the best medium for stratification and post dormancy development of seedlings. Cytokinins in the testa, mesocarp and embryos were extracted at various stratification periods using TLC techniques. At least four cytokinins were identified and are the first reported in this species.

Both *in vitro* and *in vivo* application of 0.1 and 2 mg/l of BAP respectively, were able to release embryo dormancy more rapidly. For these treated embryos the rate of cotyledon greening, growth of hypocotyl, roots , shoots and its germination percentage were determined after incubation at 20°C in continuous light for one month.

A micropropagation system for rowan was successfully developed. This study demonstrated that BAP alone or in combination with IBA were successful in inducing shoot proliferation from nodal segments and shoot tips of 2 and 8-month old aseptically produced seedlings. The shoots produced could be further recycled, proliferating more shoots. The best levels of BAP that stimulated multiple shoots production were BAP at 0.2 mg/l in combination with either 0.01 or 0.02 mg/l of IBA. Good rooting of these shoots were obtained by cutting individual explants measuring <1 cm and transferring them to a rooting medium supplemented with either 0.01, 0.05 or 0.10 mg/l of IBA or IAA. However, *in vitro* produced shoots of rowan were found to be easier to root than those produced *in vivo* and produced a higher rooting percentage by using a commercial rooting powder; Seradix 3. It has several advantages as aseptic techniques are not required and more shoots could be rooted in the covered propagator trays in the green house making sure the atmosphere inside the propagators were humid enough to avoid dessication. Two weeks were needed for hardening.

SECTION I

INTRODUCTION

1. GENERAL

Broadleaved woodlands are a dominating feature of much of Britain's landscape. In the past they were the principal source of timber for building, fencing and fuel; today they continue to supply half the country's consumption of hardwoods (Evans, 1984). There are exactly 2000 kha. of woodland in Britain of which 660 kha. are broadleaved woodland (Steele and Peterken, 1982). Forest trees, of all the crops cultivated by man, have perhaps received the least attention from researchers. Yet the potential for improvement is great and in Britain, as in many other countries foresters are increasing their efforts in this direction. Nevertheless,little detailed research has been performed on the seed biology of many deciduous trees (Gordon and Rowe, 1982). In particular, little is known of the genetical, environmental, physiological and biochemical differences in seed dormancy between different tree and shrub species. Few studies have been performed on the biochemical changes that take place in the majority of tree and shrub seeds during their release from dormancy. The understanding of the molecular basis of seed dormancy is so fragmentary that there is no plant species for which there exists an adequate account of its dormancy in cellular and molecular terms (Bradbeer, 1988). In contrast to the conifers, little detailed practical work has been undertaken in the broadleaved species, towards improving nursery germination. Even less work of a basic nature has been undertaken relating the practical treatments employed with the physiological and biochemical processes involved (Gordon and Rowe,1982).

World shortage of tropical hardwoods.

Hardwoods, that is, broadleaved non-coniferous trees, predominate in the tropical regions of the world. Hardwood products contribute most to world trade. Increased population in developing countries and pressure for land for cultivation, however,has led to deforestation at an alarming rate (Wardle, 1982). In many areas this has had harmful consequences on the environment. Forests that are especially threatened are those which are not adequately protected or where conversion to coniferous species is practiced for a higher economic return.

In recent years the total volume of hardwood logs in world trade has varied between 42 and 49 million m^3, 95% of such logs originate from tropical countries, of which Malaysia and Indonesia together account for 80% of world exports. Peninsular Malaysia is predicted to experience a shortage of timber to meet domestic consumption by the mid-1990's (Chong, 1979). This drastic transition from being a leading exporter to a net importer of tropical hardwood will incur serious socio-economic repercussions to the nation as a whole. To avert this impending crisis a programme of planting (The Compensatory Plantation Programme) has been launched. The programme aims to establish 188,200 ha of fast growing hardwood plantations (Chong, 1984). Sarawak, meanwhile has undertaken large scale planting in an effort to reafforest large areas of degraded forest that has resulted from logging and shifting cultivation agriculture (Lee, 1981). It marks a new era in Malaysian forestry in which timber will no longer be a natural,but rather a man-made resource. The increased demand for such a renewable resource provides a challenge for research on forestry and forest product technology, with the aim of increasing the supply of such products. An in-depth knowledge of seed

technology is essential in order to back up such a large-scale reafforestration programme.

Methods of processing timber seeds, as routinely as is presently the case with agricultural seeds, needs to be developed.Most tropical hardwood species in Malaysia belong to the family Dipterocarpaceae. Such woods make up about 25% of the world trade in hardwood (Ashton, 1980). One of the major problems in the propagation of these indegenous species is lack of seed and seed production. Dipterocarp seed is produced on trees only on average once every 5 to 15 years so it is almost impossible to achieve a constant supply of planting stock from seedlings. Moreover, such seeds have a short viability period (Sasaki, 1980) and like many primary forest species storage by conventional means is problematic. This is not a problem confined to dipterocarp species,many forest trees, eg. tree legumes do not regularly bear fruit,and when they do, bear seed of low quality (Sasaki *et.al.*,1978). Also, premature abortion of the seed is common because of frequent insect and fungal attack (Daljeet Singh, 1976). It is therefore difficult to predict the yield of remaining viable seeds. Successful vegetative propagation of these species either by stem cuttings or tissue culture techniques could therefore be an important alternative method of seedling production. With the above problems as a basis for future research , it was thought desirable to design an extensive study of a local U.K. broadleaved tree species whereby various techniques of seed technology and micropropagation could be acquired. This experience would be directly applicable to broadleaved tree seed problems in either the developed or developing world.Thus the species *Sorbus aucuparia L.* was chosen because little was known about the nature of seed dormancy

and, in addition, extensive work on the micropropagation of this species was lacking. As a result the work described here comprise two distinct lines of research on the above species. The first is a study of seed propagation in particular factors that affect after-ripening and dormancy breaking and the second is the development of a micropropagation system from a seed based starting material.

2. LITERATURE REVIEW

2.1 Some aspects of seed biology of *Sorbus aucuparia* L.

(i) Taxonomy, distribution and variation

Rowan (*Sorbus aucuparia L.*) is a member of the *Rosaceae* family, a deciduous tree which grows to between 9 and 18 metres high.It has a fairly quick erect growth when young, becoming more spreading and graceful with age . It is also called the **Mountain Ash** because of its ash (*Fraxinus excelsior* L.)-like compound leaf together with its ability to survive in upland regions although the two trees have no other features in common (Mitchell, 1985).

It is native to Britain, although less common in the central English counties. In the past,it was widely planted in the Scottish Highlands because of a widespread belief that it would protect dwellings from witchcraft. The name `**rowan**' is derived from the Norse `ron', which is related to the word 'rune', for the Norsemen used to carve their runic alphabet on rowan wood or on stone (Mitchell, 1985). Rowan is very hardy and is the most common deciduous tree found at altitudes above 1000 m. It grows on land ranging from bleak moorland to open wood, but is a light demanding pioneer and cannot tolerate shade, although it may sometimes form an understorey in old Scots Pine woods. Rowan grows best on light textured brown earths and more fertile peat but does not tolerate waterlogged conditions (Evans, 1984).

Rowan may be found growing in North America, from Iceland to North Russia, in the mountains of Central Spain, Portugal, Corsica, Italy, Morocco and Northern Asia Minor. In North Asia rowan extends in a continuous belt through Siberia to the far-eastern coast (Gillham and McAllister, 1977).

Sorbus is a plastic genus comprised of poorly defined taxa (Calder and Taylor, 1968) and *S. aucuparia L.* may form a distinct geographic race as evidenced by its wide distribution within which several forms and varieties are known (Harris and Stein, 1974).

Sorbus sect *aucuparia* has been broadly reviewed by Gillham and McAllister (1977) and they report that there has been a lot of errors in naming the trees by horticulturists. Hybrids between species are known to occur due to the indiscriminate use of seeds as a means of propagation. However, this only applies to the sexually reproducing diploid species with a chromosome number of 2n = 2x = 34, which includes *Sorbus aucuparia L.* All tetraploids with a chromosome number of 2n = 4x = 68 are probably apomictic and will therefore come as true from seed as from a graft or cutting (Gillham and McAllister, 1977).

(ii) Phenology and reproductive material

Leaves are pinnate 12 - 23 cm long with 6 or 7 pairs of leaflets. The leaflet edges are distinctly toothed and the leaves and buds are alternately set on twigs and not oppositely set as in Ash. Its winter buds are exceptionally large, dark purple in colour, and terminate in a peculiar one-sided point; each scale is tufted with white hairs (Mitchell, 1985).

(iii) Flowering and fruiting

The flowers are unpleasantly scented approximately 8 mm in diameter and exhibit the structure typical of Rosaceae. The small, white perfect flowers are numerous and densely packed in large, rather flattened clusters to form a corymb (7 - 12 cm across) from May until June

depending on location. Each individual flower possesses five green sepals, five white petals, nectaries to attract bees, a large number of stamens and a central pistil composed of three to four carpels. Calyx and flower stalks are covered with hairs. They are pollinated by many kinds of insect attracted by its scent and nectar (Laidlaw, 1960). Another characteristic feature of *Sorbus* is the grouping of the flowers and their resultant fruit, into clusters that are made up of one main stalk and many smaller ones.

The fruits (Plate I.1a and c) which is somewhat barrel-shaped, ripen from July until September and are bright red in colour. The fruit is a two to five-celled berry-like pome. Within each cell are one or two small brown seeds (Plate I.1b).

The seed of *S. aucuparia* belongs to mesotestal group of seed (Corner, 1976) with the mechanical layer in the mesophyll. The mesophyll of the mechanical layer composed of 4 layers of cuboid sclerotic cells. Its outer epidermis is mucilaginous when wet which disappears if washed. In this thesis for simplicity the mechanical mesophyll cells is termed the **testa** (Fig. I.1). Underneath the testa is the mesocarp essentially consisting of of 2 - 3 layers of enlarged cells with thin inner brown walls.

Rowan berries remain on trees until late winter and are consumed by birds during this period. The hard small seeds pass through bird's digestive systems and are deposited often at a distance from the trees that grew them (Harris and Stein, 1974). Rowan trees start to bear seed at about 15 years of age and good seed crops occur almost annually (USDA Forest Service, 1948). Seeds are subject to attack by several species

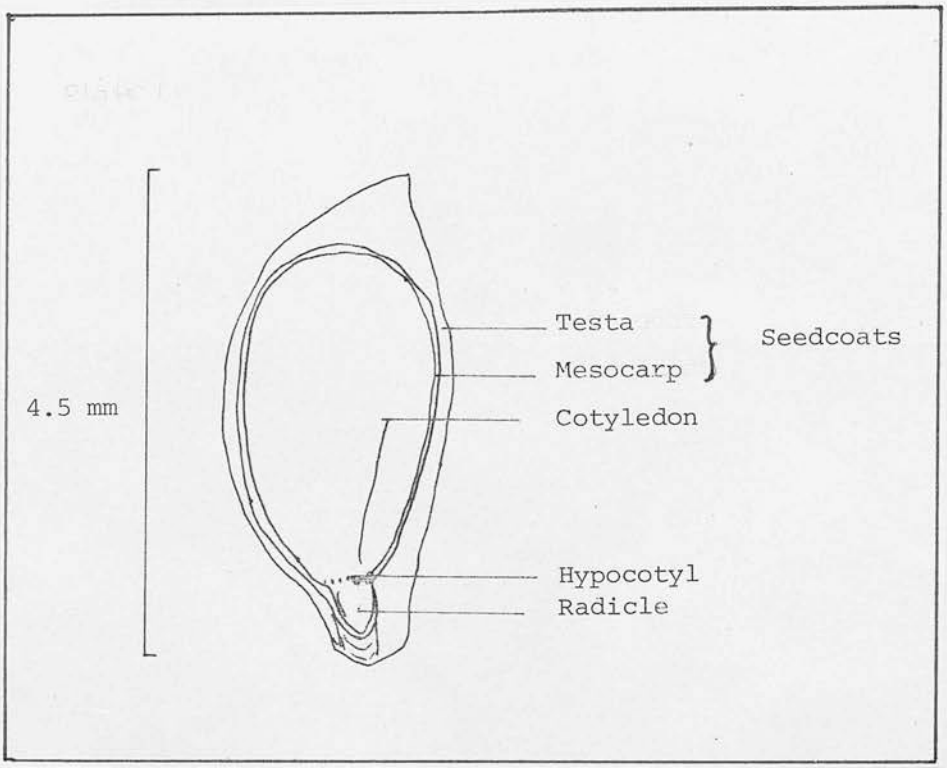
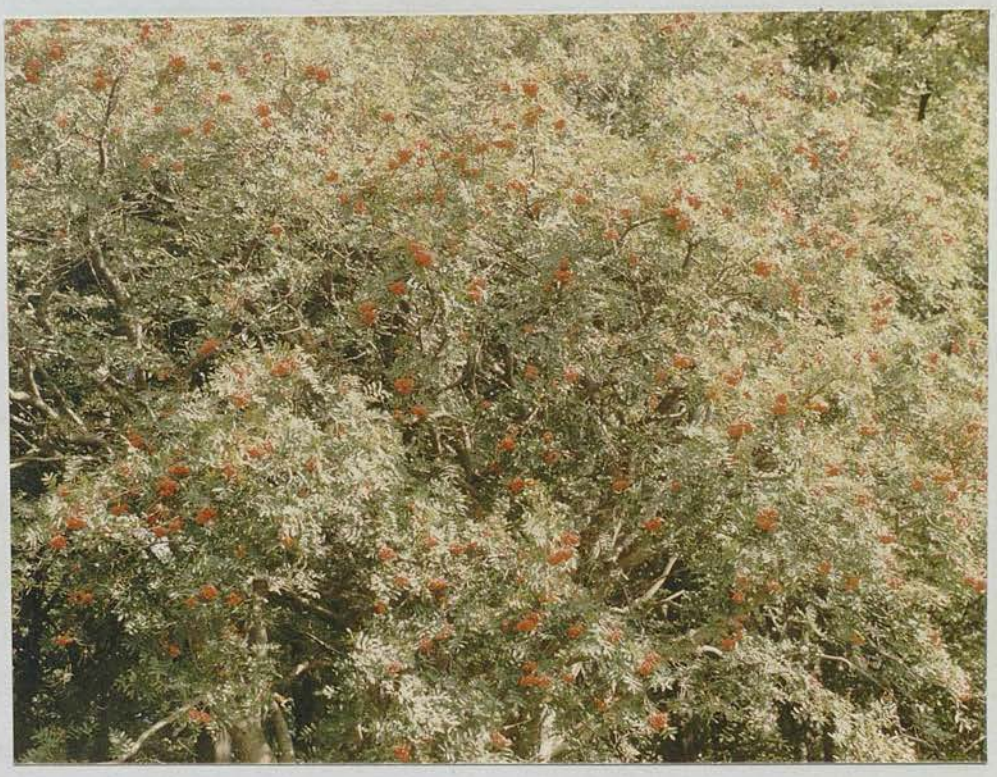


Fig. I.1 : ***Sorbus aucuparia*** **L. Rowan or Mountain-ash longitudinal section through a seed x 14.5**

a

b



c

Plate I.1 : (a) Ripe fruits of rowan (*S. aucuparia* L.) x 1 cm.

(b) The seeds of *S. aucuparia* L. Scale in mm.

(c) Matured fruits during late Autumn

of **Chalcid flies** (Rohwer, 1913).

(iv) Fruit collection, seed extraction and storage

The fruit must be picked or shaken from the tree as soon as it begin to colour (Shoemaker and Hargrave, 1936) to prevent losses to birds. Seeds are normally extracted from the fruit and sown immediately after collection, or the seeds may be dried and stored before pretreating for spring sowing.

Cleaned seeds have been stored under cold dry conditions for between 2 to 8 years with little loss of viability (Flemion, 1931, Heit, 1967; Rehder, 1940). For best results, storage in sealed containers at 6 to 8 % M.C. and temperature 3 - 5 °C is recommended (Heit, 1967). Storage at room temperature has proved satisfactory for *S. aucuparia* but relative humidity much above or below 25% are unfavourable at such storage temperatures (Flemion, 1938).

Dormancy is broken by pretreating the seeds in a compost medium for 2 weeks at between 20 - 25°C followed by 14 - 16 weeks at 1-5°C.

Seeds are usually sown before mid-April to avoid inducing secondary dormancy , due to high seedbed temperatures (Gordon and Rowe, 1982).

(v) Utilization

As rowan trees are particularly hardy they often form a significant component of upland shelterbelts (Evans, 1984). Its wood is good for turning and carving since it has a dense hard timber,it also makes good firewood. It is also extensively utilised as an amenity tree.

2.2 Seed dormancy of broadleaved trees

There has been much discussion in the literature regarding the definition of the term 'dormancy' (Vegis, 1964; Amen, 1968; Wareing, 1969). A comprehensive survey of the literature of seed dormancy may be found in the second volume of the monograph by Bewley and Black (1982). Dormancy can be defined as any condition of the viable seed or plant organ that makes it resistant to germination under environmental conditions that are otherwise favourable (Ellis *et.al.*,1985). The term 'dormancy' and 'quiescence' must be differentiated. A quiescent seed will not germinate because environmental conditions are unfavorable , on the other hand, given the correct environmental conditions a seed will germinate immediately. A 'dormant' seed however, will germinate only after the application of some triggering substance or factor or after some specific environmental stimulus, which need not be necessarily be constant.

2.2.1 Causes,occurrence and classification

There are several ways of classifying the mechanism of dormancy but none appears to be entirely satisfactory (Duffus and Slaughter, 1980) mainly because the underlying causes of dormancy are still unknown. The most prominent accounts are by Barton (1965), Nikolaeva (1967,1977), Villiers (1972), Khan (1977), Harper (1977), Bewley and Black (1982), Mayer and Poljakoff-Mayber (1982)and the most recently Bradbeer (1988). In many cases dormancy in the seed may reside in two distinct sites, namely the embryo coverings and the embryo itself, often therefore called either coat-imposed or embryo dormancy respectively. If a dormant seed contains an embryo which proves to be capable of germination after isolation, it may be concluded that the cause of dormancy resides in the seedcoats

which enclose the embryo. However, in many dormant seeds there is a **double dormancy**, caused by the combination of both the seed coverings and their embryos. The difference between coat-imposed and embryo dormancy can sometimes be ill-defined especially in seeds which have a chilling requirement, since many of these seeds pass from a state of embryo dormancy to one of coat-imposed dormancy during the course of a chilling period (Wareing, 1982).

2.2.1.1 Classification following Nikolaeva (1977)

Nikolaeva (1967;1977) divided dormancy types (in temperate tree species) into three groups: **exogenous, endogenous** and **combined**, depending on the relationship between the factors inhibiting germination and the conditions required to eliminate the block (Table I.1).

(i) Exogenous dormancy (A)

Exogenous dormancy types are those which are caused by physical or chemical properties of the outer seed coverings which can be overcome by various physical methods.

Physical dormancy (Aph) or hardseededness is the most commonly recognised type. This phenomenon is typical of many plant species belonging to a number of families (*e.g. Papilionaceae, Malvaceae, Chenopodiaceae, Liliaceae*). Seeds exhibiting this condition have hard seedcoats which may contain cutinised layers preventing the passage of water into the embryo. Most of woody legumes exhibit this type of dormancy mechanism as in *Cytisus spp., Laburnum spp., Robinia spp.*(Gordon and Rowe, 1982); in these cases it is the sole factor that prevents germination.However, in *Tilia spp.*, the hardseeded condition

Table I.1: Classification of broadleaved tree seed dormancy (modified from Nikolaeva, 1977) by Gordon and Rowe (1982)).

TYPES OF DORMANCY	FACTORS CAUSING DORMANCY	CONDITIONS BREAKING DORMANCY	EXAMPLES
A- EXOGENOUS DORMANCY			
Physical (Aph)	Impermeability of seedcoat to water	Scarification	*Robinia pseudo-acacia*
Chemical (Ach)	Presence of inhibitors in pericarp	Removal of pericarp or leaching	*F. chinensis* var. *rynchophylla*
Mechanical (Am)	Mechanical resistance of covers to embryo growth	Various methods for destroying the covers	*Eleagnus angustifolia*
B and C - ENDOGENOUS DORMANCY			
Morphological (B)	Underdevelopment of embryo (UE)	Warm moist treatment	(occurs only in combination with other factors) *Elaeis guineansis*
Physiological (C)	Physiological inhibiting mechanism (PIM) of germination		
Shallow (C_1)	PIM weak	Short chilling treatment, growth stimulators, dry storage	*Betula pubescens*
Intermediate (C_2)	PIM intermediate	Long chilling treatment only and several other influences such as GA's[1]	*Nothofagus obliqua*
Deep (C_3)	PIM strong	Long chilling treatment only	*Sorbus aucuparia*
B and C - MORPHO-PHYSIOLOGICAL			
	Combination of UE and PIM		
Deep (B - C_3)	Combination of UE and strong PIM of germination	First warm, then cold treatment	*F. excelsior*
Deep, epicotyl (B - Ce_3)	Combination of UE & strong PIM of epicotyl growth	Same	*Viburnum opulus*

[1] GA's = Gibberellic acids

where the seedcoat is impermeable to water and gases (Aph) merely exacerbates a second dormancy mechanism where the embryo possesses a deep physiological dormancy(C_3).

Chemical dormancy (Ach) particularly of dry indehiscent fruits is caused by the presence of water-soluble inhibitors in their pericarps. It is said that chemical dormancy occurs regularly in combination with other dormancy mechanisms, but the presence of inhibitors may be obscured or leached out unnoticed or are neutralised during the lengthy treatments used to overcome the other, stronger dormancy controls (Nikolaeva,1967; Gordon and Rowe, 1982).

Mechanical dormancy (Am) is similarly most often exhibited in combination with other mechanisms of the types of endogenous and exogenous dormancy (Nikolaeva, 1977) but, unlike the chemical dormancy it cannot be masked. It is due to the tough seed coverings that often provide a mechanical constraint to embryo growth (hazel nut shell or plum stone etc.). In seeds of *Tilia spp.*, physiological dormancy is linked with hardseededness (Nikolaeva,1977).However, dormancy caused by the seed coats is much more difficult to overcome than the hard-seeded condition and its removal takes longer such as found in *Crataegus spp.*and *Carpinus spp.*(Gordon and Rowe,1982).

(ii) Endogenous dormancy (B,C)

This dormancy is extremely difficult to remove and occurs in many forms. It is determined primarily by the conditions of the embryo itself, separated by Nikolaeva into three main sub-groups : *morphological dormancy* (B), *physiological dormancy* (C) and *morpho-physiological dormancy* (B-C). These endogenous dormancy types can only be broken by

treatments which bring about physiological changes such as light, stratification at certain temperatures and the application of plant growth regulators.

Seeds with *morphological dormancy* (B) are dispersed with underdeveloped embryos which must complete their growth and development before the seed can germinate, examples are *Fraxinus excelsior* (Villiers and Wareing, 1964), *Magnolia spp.* and numerous representatives of *Palmae, Araliaceae, Magnoliaceae, Ranunculaceae* and so on. Seed exhibiting morphological dormancy are more frequent in the tropics (Nikolaeva, 1977). In most cases, however, the seed stays dormant even after embryo growth is completed. This is due to morphological dormancy being associated with the physiological dormancy.

Physiological dormancy of seeds (C) is due to decreased embryo activity which, together with a restriction to gas exchange imposed by the seed covers, produces what Nikolaeva (1977) called the physiological inhibiting mechanism (PIM). This term PIM probably includes a wide range of different dormancy control mechanisms. Although there are borderlines between them, three groups and strengths of PIM can be recognised, namely, shallow (C_1), intermediate (C_2) and deep (C_3) physiological, dormancy.

Shallow dormancy (C_1) is typical of freshly collected seeds of many species from the temperates zones as in *Betula spp.*(Gordon and Rowe, 1982). This dormancy may disappear gradually in the course of after-ripening during dry storage of the seeds . It can also be removed more quickly by : (1) short treatments of moist prechilling, (2) applications of various PGR's, (3) the illumination conditions in the laboratory, (4)

increasing the partial oxygen pressure, or (5) damaging the seed covers (Gordon and Rowe, 1982). However, the most practical nursery treatments according to Gordon and Rowe, (1982) would be to rely on the efficacy of a short moist prechilling treatment (2 - 4 weeks) as in some seed lots of *Betula spp.* or an application of a PGR , such as gibberellin (GA).

Intermediate dormancy (C_2) is more complicated. This type of physiological dormancy is observed in the seeds of for example, *Acer nugundo, Fraxinus pennsylvanica* and *Amaranthus spp.*(Nikolaeva, 1977). The excised embryos will normally germinate when placed in ideal conditions notably if the seeds have been exposed to dry storage for some time (Gordon and Rowe, 1982). However, the entire seeds normally require a sufficiently protracted moist prechilling treatment (4 - 12 weeks) before it can germinate, although GA application may stimulate germination (Nikolaeva, 1977; Gordon and Rowe, 1982).

Deep physiological dormancy (C_3) is recognised in embryos that grow into abnormal 'physiologically stunted' or retarded growth even under the most favourable conditions. A prolonged moist chilling treatment (more than 12 weeks) at 1 to 7°C is necessary to break the dormancy and no other treatment is successful (Nikolaeva, 1977). This deep physiological dormancy is especially common among Rosaceous species in general such as *Sorbus spp., Malus spp., Chaenomeles spp.* (Nikolaeva, 1977; Gordon and Rowe, 1982).

In woody plants, two forms of *morpho-physiological dormancy (B-C)* are of importance, namely, (1) *morpho-physiological hypocotyl dormancy* and (2) *morpho-physiological epicotyl dormancy* (Gordon and Rowe, 1982). *Fraxinus excelsior* (Ash) seeds exhibit the former type ($B-C_3$). Ash seed

is normally dispersed with an immature embryo, which needs a period of warm-moist treatment to fully grow to maturity. It then requires a cold moist treatment to remove physiological dormancy (Villiers and Wareing, 1964). Whereas, with morpho-physiological epicotyl dormancy (B-Ce_3), it can be removed by the same alternating warm and cold treatment but in this case the radicle emerges during the warm period and the epicotyl or shoot-bud only outgrows after the seedling (with a sufficiently developed root system) has been subjected to a chilling treatment. An example of a species which shows this particular type of dormancy is *Viburnum opulus* (Nikolaeva, 1977).

A great number of the tree species produce seeds exhibiting combined dormancy, characterised by various combinations of the exogenous and endogenous dormancy types. *Tilia cordata* seeds possess a physiological dormancy (C_3) which is linked with a hard coat (A_{ph}) (Gordon and Rowe, 1982). *Tilia platyphyllos* seeds have a similar dormancy mechanism (Nagy *et al.*,1981).

2.2.1.2 Classification following Bradbeer (1988)

According to Bradbeer (1988) dormancy in seeds occurs primarily through one or a combination of the mechanisms included in Table I.2.

A.Dormancy caused by the embryo coverings

A.1 Restriction of gaseous exchange. Bradbeer (1988) found no convincing evidence in the literature that it would bring about dormancy. There is no doubt that the embryo coverings provide a barrier to gaseous exchange and it has yet to be proven that such a limitation can cause a seed to be dormant.

Table I.2 : The main identified mechanisms of seed dormancy (after Bradbeer, 1988)

A.Dormancy caused by the embryo coverings (pericarp, testa, perisperm and endosperm)

1. Restriction of gaseous exchange
2. Restriction of water uptake
3. Mechanical restriction of embryo growth
4. Water-soluble inhibitors in embryo coverings
5. Dormancy from the failure to mobilize extra- embryonic food reserves.

B.Embryo dormancy

1. Underdeveloped and undifferentiated embryos
2. Block to nucleic acid and protein synthesis
3. Failure to mobilize food reserves of embryo
4. Deficiency of plant growth substances
5. Presence of inhibitors

A.2 Restriction of water uptake

As embryo size increases after imbibition, this should be easily recognised. However, in some hard seeds it is difficult to distinquish between the restriction of water-uptake and the mechanical restriction of embryo expansion as the cause of the dormancy. This may be solved by studying the diffusion of labelled water (^3H_2O) into the seeds by means of autoradiography, as detailed by Jackson and Variano Marston (1980).

A.3 Mechanical restriction of embryo growth

The seed coats often form mechanical restriction to embryo growth. In *Alisma plantago*, the embryo coverings resisted the full imbibition of its embryo so that its seed with a partly swollen embryo could lie for years in an aquatic environment, unable to germinate (Crocker and Davis, 1914). This type of physical restriction perhaps can be overcome by natural events that gradually weakens the seed coverings or it may also be ruptured by continued development of the embryo. This implies that there are two aspects of this form of dormancy, the mechanical restriction of the coverings and the physiological ability of the embryo to overcome the restriction. In another example, Esashi and Leopold (1968) showed that, the dormant upper seed of *Xanthium* has a radicle unable to develop enough force to break through the testa. In hazel nuts, the pericarps were split at the margins of the two carpels by the expansion of the embryo, mainly the cotyledons. In *Eucalyptus pauciflora* and *E. delegatensis*, the primary cause of dormancy was the mechanical resistance of the seed coat (Bachelard, 1967). He concluded "that the germination processes was first initiated and can develop to a certain stage in imbibed intact seeds but when resistance to germination is encountered, in this case by a mechanically resistant seed coat,further stages of germination are prevented".

A4. Water soluble inhibitors in embryo coverings

Bradbeer, (1968) inferred that the papery testa of hazel (*Coryllus avellana*) seeds contained water-soluble inhibitors which checked the germination of the non-dormant newly harvested embryo upon imbibition. A summary of the best examples in which identified water-soluble

inhibitors have been demonstrated to occur in the embryo coverings of four species of broadleaved trees is given in Table I.3.

Abscisic acid (ABA) is involved in all four of the species, in all cases exogenously supplied ABA to non-dormant seed has been shown to inhibit germination (Bradbeer, 1988).Furthermore, the reported ABA concentrations in the seed coverings appears to be high enough to maintain embryo dormancy although such inhibition is reversible. This may be due to the enzymic inactivation of ABA, from leaching or from a change in the sensitivity of the embryo to ABA, or from the accumulation of germination promoters (Bradbeer, 1988).

A5. Dormancy from the failure to mobilize extra-embryonic food reserves.

The mobilization of food reserves in the endosperm or the perisperm is either not detectable, reduced or absent in a dormant seed (Bradbeer, 1988). Stokes (1953a) reported that there was no germination of *Heracleum sphondylium* endosperm at 15°C because hydrolysis of reserve proteins did not occur at that temperature, although there was some hydrolysis of reserve polysaccharides. He subsequently showed that isolated embryos germinated and grew normally in a culture medium containing 2 % glucose, essential minerals and an appropriate nitrogen source (Stokes, 1953b). More importantly, however, normal germination of the dormant seed occurred in seed chilled at 2°C, in addition, in this seed the endosperm protein was found to have been hydrolysed. Stokes (1953a) inferred that at 2°C a product of embryo metabolism diffused into the endosperm which brought about protein hydrolysis. Failure to germinate may be regarded as a block to the mobilization of extra-

Table I.3 : Water-soluble germination inhibitors shown to occur in embryo coverings and embryos of dormant seeds (extracted from Bradbeer, 1988).

Species	Substance	Location and concentration (nmoles/gm dry wt) in fruit and seed	Source
1. *Malus sylvestris* (apple)	ABA[1,3]	Testa	Pieniazeck and Grochowska (1967)
2. *Corylus avellana* (hazel)	ABA[2,3]	Pericarp (1.4) Testa (19) Embryo (0.09)	Bradbeer (1988)
3. *Fraxinus americana* (white ash)	ABA[2,3]	Pericarp from samara (2.8) Dormant seed (1.7)	Sondheimer *et.al.* (1968)
4. *Acer saccharum* (sugar maple)	ABA[2,3]	Pericarp (0.83) Testa (5.1) Cotyledons(1.9) Axis (2.8)	Enu-Kwesi and Dumbroff (1978)
"	Ferulate[1,4]	Pericarp (67) Testa (124) Cotyledons (21) Axis (216)	Enu-Kwesi and Dumbroff (1980)
"	o-Coumarate[1,4]	Pericarp (55) Testa (329) Cotyledons (43) Axis (348)	"
"	p-Coumarate[1,4]	Pericarp (189) Testa (713) Cotyledons (244) Axis (799)	"

Key : 1. Provisional identification
2. Unequivocal identification by physico-chemical method.
3. Inhibits germination of non-dormant seeds of the species.
4. Does not inhibits germination of non-dormant seeds of this species.

embryonic reserves, however, the cause of this may be due to a failure of the embryo to secrete an activating factor.

Considering the case of *Heracleum sphondylium*, the embryos which require basic nutrients in order to germinate are not categorised as dormant. But due to the incapability of the embryos to mobilise the extra-embryonic food reserves, the causal factor for the dormancy must lie within the embryo.

B. Embryo dormancy

In general, mechanisms of embryo dormancy are ill-defined. Elucidation at a molecular level are lacking although group mechanisms have been proposed by Bradbeer (1988), (Table I.2).

B1. Underdeveloped and undifferentiated embryos.

In the case of *Heracleum sphondylium* (Stokes, 1952) seeds possess a relatively small embryo at the time of dehiscence, so that a marked period elapses between imbibition and germination. Whereas in *Fraxinus nigra* (Steinbauer, 1937) and *F. excelsior* (Villiers and Wareing, 1964), embryos are fully differentiated when shed but are a third to a half shorter in length than those just prior to being germinating ripe. Embryo growth in such seeds is favoured by a temperature of 20 - 30°C but a 2 - 3 months chilling period at 5 - 10°C is required before germination will occur. It has been shown in hazel, *Corylus avellana* (Jarvis *et al.*, 1978) that considerable embryonic axis growth and development from dormant hazel embryos will occur when cultivated in an organic medium supplemented with 2 % sucrose. They concluded from this work that the radicle and plumule of dormant hazel embryos were not intrinsically dormant. The immediate cause of hazel embryo dormancy must

be an inability to mobilize the reserve materials of the embryos. Hazel embryos dormancy can be broken by exogenous application of GA_3 or by a treatment which results in the synthesis of endogenous GA (6 weeks chilling at 5°C followed by transfer to a higher temperature, 20°C), after which embryonic reserves are mobilised. Imbibed dormant embryos show no detectable mobilisation of reserves. Hence in hazel embryos, dormancy may be a result of an insufficiency of GA synthesis or an inability to release bound GA, or the rapid metabolism of free GA in the embryo. There is evidence that the embryonic axis of hazel seed is the main site of GA synthesis although there may be some synthesis and release of GA by the cotyledons (Arias *et. al.*, 1976).

In general a molecular explanation of seed dormancy is lacking. It is possible that the immediate cause of embryo dormancy is not a deficiency of a protein, but rather the regulation of enzymes activity or changes on membrane permeability or membrane transport (Bradbeer, 1988).

B2. Endogenous inhibitors in embryo dormancy.

There are few firmly established cases of the presence of inhibitors located in dormant embryos (Table I.3). However, ABA has been identified as the inhibitor in apple, *Malus sylvestris* (Le Page-Degivry & Bulard, 1979); Yew, *Taxus baccata* (Le Page-Degivry, 1973), and lettuce, *Lactuca sativa* (Speer and Tupper, 1975) embryos.

The use of Gas Layer Chromatography / Mass Spectro- photometry (GLC/MSP) to identify suspected inhibitory substances also permits the accurate estimation of its concentration, for example, the concentration of ABA that has been determined for *Acer saccharum*, sugar maple, (Enu-

Kuwesi and Dumbroff, 1980) is 3 x 10^{-6}M, whereas for apple the concentration was 3 x 10^{-7}M (Le Page-Degivry and Bulard, 1979) and 1 x 10^{-7}M for hazel (Bradbeer, 1968). ABA may be present in both free (Le Page-Degivry, 1979) and bound (Corvillon and Martinez-Honduvilla, 1980) forms as exemplified by *Malus* sylvestris (apple). The bound form may be hydrolysed *in vivo* to give the free form. There is a reasonably strong indirect evidence that ABA maintains an embryo in the dormant state, eg. the concentration of ABA in the embryo and embryo coverings of sugar maple fell during chilling at 5°C (Enu-kuwesi and Dumbroff, 1978). However, Villiers and Wareing (1965) working with ash (*F. excelsior*) found that chilling , did not remove the inhibitor from either the seed or the embryo. In this case breaking of dormancy may involve a change in sensitivity to the inhibitor (Bradbeer, 1988).

B3. The possession of more than one dormancy mechanism.

Some seeds and fruits possess multiple dormancy mechanism eg. cocklebur, *Xanthium pennyylvanicum*, (Wareing and Foda, 1957; Esahi and Leopold, 1969), ash, *F. excelsior*, (Villiers and Wareing, 1964) and hazel, *C. avellana*, (Bradbeer *et al.*, 1978). It is not known whether more than one biochemical block may operate in these cases. Possibilities include dormancy operating through the pericarp or seed coverings, particularly by means of chemical inhibitors located in these structures. In addition, dormancy may be imposed by impermeable seed coats or mechanical resistance. It has also been speculated that a deficiency of nucleic acid or protein synthesis may result in a failure to mobilize food reserves, which may in turn be due to a lack of plant growth promoters or to the presence of inhibitors (Bradbeer, 1988).

2.2.1.3 Secondary dormancy

Various type of dormancy can be recognised called **primary**, *innate* and *internal* ; **secondary**, *induced* and *enforced* according to when or how they came to be present in seeds (Khan, 1980/1981). **Primary dormancy** aptly describes dormancy that prevents germination during development and maturation on the mother plant (Khan, 1980/1981) and usually also for some time after shedding of the seeds (Karssen, 1980/1981). **Secondary dormancy**, however, describes seeds develop dormancy naturally or artificially (Khan, 1980/1981) after dispersal or harvest but are primarily non-dormant or have emerged partly or fully from primary dormancy (Karssen, 1980/1981).

In general, secondary dormancy is promoted by the imposition of environmental constraints, although no single factor can generally be held responsible for the inhibition of germination (Karssen, 1980/1981). These factors like temperature, lack of light or oxygen, presence of inhibitors and moisture conditions may all contribute to impeded germination and thereby enable the induction of secondary dormancy. Among these, temperature is an important rate determining factor (Karssen, 1981). Various investigators have found that secondary dormancy can be induced in seeds which possess inherent deep dormancy such as in *Cornus florida*, dogwood; *Sambucus canadensis*, elder (Davis, 1927); *S. aucuparia*, rowan, (Flemion, 1931), *Rhodotypos scandens*, black jetbead, (Flemion, 1933), and *Malus spp.*, apple (Visser, 1954). In such seeds secondary dormancy occurs if the process of cold stratification is interrupted by a period of a higher temperature; Visser (1954) found that this was accompanied by a sharp increase in the respiration rate. But when the seeds were immersed in water the interruption did not lead

to a rise in respiration and did not cause any secondary dormancy (Nikolaeva, 1967). Nikolaeva suggested that during low temperature conditioning a 'germination factor' was formed in embryos which was easily destroyed by a high oxygen concentration. The presence of seed coverings which prevents entry of oxygen permits the perpetuation of this factor even at high temperature. Tissaoui and Come (1973) reported that the embryo dormancy in apple could be overcome by a treatment for a week in an oxygen-free atmosphere at room temperature. This finding seems to agree with Nikolaeva's hypothesis with regard to the existence of an oxygen-activated compound which impeded germination. In addition, it appears that these seeds as long as they exhibit secondary dormancy, can retain their viability for a long time, even when fully imbibed.

The phenomena of secondary dormancy is poorly understood but has been reviewed by Karssen (1980/1981), a number of mechanisms of secondary dormancy induction have been put forward. These include; a) changes in light requirement, b) effect of temperature (high or low), c) Phytochrome(Pfr)-requirement and membrane properties, d) changing sensitivity to growth regulators, and e) metabolic factors.

According to Karssen (1980/1981) ,no specific relationship has been found between the induction of secondary dormancy and any particular metabolic event in seeds. Therefore, secondary dormancy in seed reflects a general insensitivity to external and possibly internal inducers of germination. Studies on changing light sensitivity indicates that changes in membrane organisation may be involved (Hendricks and Taylorson, 1978).

2.2.1.4 The breaking of seed dormancy.

Seed dormancy of a particular species can often be overcome by treating the seed in any one number of different ways (Barton, 1965a; Stokes, 1965 and Black, 1980/81). According to Bradbeer (1988) the majority of dormant seeds require the application of one or more environmental factor(s), not necessarily those that would be required for germination, before it can be broken. Methods to break seed dormancy can be divided into two; (i) Exposure to a constant single factor eg. *chilling , dry storage, elevated temperature, light , scarification* (ii) Exposure to fluctuating conditions, and particularly temperature and light .

(i) Dormancy breaking in seeds with a chilling rquirement.

Pretreatment of imbibed seeds at a low (1 - 5°C), but not freezing, temperature over a period of time can be extremely effective dormancy-breaking treatment in many seeds and is widely applied in practice. The period of exposure to this condition varies depending on the species and depth of dormancy it possesses. This process is also called **"stratification"** because in many cases the seed materials is placed in alternate layers of sand or soil.

Seeds which require chilling to overcome dormancy, often contain both growth inhibitors (Wareing, 1965) and growth promoters (Frankland and Wareing, 1966). Evidence exists to support the view that dormancy of this type is controlled by an inhibitor/promoter balance that alters when the seeds are chilled (Amen, 1968; Wareing and Saunders, 1971). The broader aspects of inhibitor/promoter interactions have been comprehensively reviewed by Wareing (1969) and Wareing and Saunders (1971).

Frankland and Wareing (1966) found that chilling caused an increase in GA content in *C. avellana* (hazel) and *F. sylvatica* (beech) seeds. Inhibitor levels, however, did not change appreciably as might have been expected. A similar response to chilling has been found in *Fraxinus excelsior*, ash (Villiers and Wareing, 1965); Kentzer, 1966). A more detailed study of GA synthesis in chilled hazel seeds was made by Bradbeer (1968; 1988) and by Ross and Bradbeer (1968, 1971 a and b). Chilling appears to remove a block to GA biosynthesis but gibberellins (GA's) are not apparently made in quantities sufficient to promote germination until the seed is transferred to a higher temperature. GLC showed the presence of a number of GAs in hazel seed extracts (Ross and Bradbeer, 1971a) GA_1 as an early formed product which is subsequently converted into other gibberellins eg. GA_4 and GA_5. Furthermore, in chilled seeds, the amount of GA per unit weight of embryonic axis is much greater than that of the cotyledons, it therefore seems likely that an early formed GA is translocated from the axis to the cotyledon, which is subsequently converted to other GAs. In apple embryos a marked peak in GA_4 and GA_7 occurred during the period of stratification (after 30 days) which was then followed by a decline in concentrations of these GA's (Sinska and Lewak, 1970). Isaia and Bulard (1978) subsequently found that at the end of a 4-month chilling period, the embryonic axis of apple seeds contained appreciable levels of GA_9 and very high levels of 'bound' GA_4 suggesting that the decline in free GA_4 observed by Sinska and Lewak (1970) may have been due to its conversion to the bound form. Similar results were obtained in *A. saccharum*, in which the transcient peak in GA activity was preceded by a transcient peak in cytokinin (CK) activity (Webb *et al.*, 1973a). In contrast, no

corresponding peaks in GA or CK activity were found in extracts of seeds of *A. pseudoplatanus* and *Pseudotsuga menzeseii*. There were, however, detectable levels of cytokinins in extracts of the radicles and cotyledons of *A. pseudoplatanus*, but these levels decline during the chilling period. In *A. platanoides* there were no significant changes in CK levels throughout the chilling period and an increase only occurred during germination. GA concentration, however, increased during chilling, before any germination had occurred (Pinfield and Davies, 1978).

There is evidence that exogenous ethylene may stimulate cells to produce their own ethylene, so effecting a positive feedback (Kende and Hanson, 1977). It is thus possible to envisage a situation in dormant embryos in which free GA's are released from a small reserve of bound GA's, thereby *priming* the pathway for GA biosynthesis in the tissue of the embryo (Wareing, 1982).

The above mechanism, however, cannot be applied to seeds where no transient increase in growth promoter is found. This suggests, in such cases, that dormancy release may involve other processes besides those affecting GA's or CK levels.

The problem of the nature of the block to GA synthesis in dormant seeds remains. The inhibitor present in many dormant seeds resembles ABA (Ross and Bradbeer, 1971b). Therefore dormancy release in seeds with a chilling requirement might depend upon reduced levels of endogenous ABA. Early studies of this problem indicate that this was the case, since seeds of both *Rosa* and *Fraxinus*, experienced a decline in endogenous ABA during the chilling period (Jackson, 1968; Sondheimer *et al.*, 1968).

Similar results have been reported for a number of other species Ash, *F. americana* (Sondheimer *et al., 1968*), sugar maple, *A. saccharum* (Webb *et al.,* 1973a), oak, *Quercus rubra* (Vogt, 1974) and apple, *M. domestica* (Rudinicki, 1969), except that in the case of pear, *Pyrus communis* no such decline was found (Dennis *et al.,* 1978). Further studies for example in tatarian maple, *A. tataricum* (Petrova and Nikolaeva, 1974); *C. avellana* (William *et al.,* 1973), *P. persea* (Bonamy and Dennis, 1977) and *M. domestica* c.v. Delicious (Balboa-Zavada and Dennis, 1977) have shown that ABA levels fall equally during low and high temperature stratification though only the former ends dormancy. The conclusion must be therefore that the experience of low temperature does not itself cause the decrease in ABA or that ABA is not involved in dormancy breaking. Hence the action of chilling in terminating dormancy is not attributable solely to an effect on ABA levels in the seeds. The process leading to dormancy release in response to chilling treatments must involve other effects besides that of a simple reduction in ABA levels, although whether a decline in ABA content is a neccessary pre-condition for dormancy release is not clear (Walton, 1980/81).

Many studies (Table I.4) have provided evidence that exogenous promoters such as GAs and CKs promote germination to some extent in some embryos and in those seeds exhibiting coat imposed dormancy(Black, 1980/81; Wareing, 1982). While GA_3 is effective in stimulating germination in large number of species, GA_7 is more active, being effective at much lower concentrations than GA_3 (Ikuma and Thiman, 1963). GA's are generally more effective than CK's at promoting seed germination, even though, CK's can convincingly nullify the effects of

ABA (Roberts and Hooley, 1988). In addition, Kinetin (K) and other CKs have the ability to reverse inhibition of germination caused by other naturally occurring inhibitors, such as coumarin and xanthatin (Khan,1980/81). K will stimulate the germination of certain seeds (Miller, 1958; Skinner and Shire, 1958; Khan, 1966). K and 6-benzylaminipurine (BAP), however, was reported to have little effect on germination by themselves but acted synergistically with light (Miller, 1958) and GA_3 (Skinner and Shire, 1958). CK's are known to both be involved in the processes of radicle elongation (Haber and Luippold, 1960; Pinfield and Stobbart, 1972) and cotyledon expansion (Ikuma and Thiman, 1963; Kursanov et al., 1969).

Table I.4: Effects of Plant Growth Regulators (PGR) on dormancy breakage of seed which normally require chilling

Species	Gibberellin	Cytokinin	Reference
Acer ginnala	-	-	Dumbroff & Webb,1970
A. saccharum	+	+	Webb & Dumbroff,1969
A. pseudoplatanus	++[1]	++[2]	Pinfield & Stobart,1972
A. pseudoplatanus	-	++	Pinfield & Stobart,1972
A. platanoides	-	-	Pinfield *et al.*,1974
Corylus avellana	+++	-	Pinfield,1967
Fagus sylvatica	++	-	Frankland & Wareing,1966
Pyrus malus	-	+	Kopecky *et al.*,1975

+++ high effect ++ medium + low - no effect

1 cotyledons 2 radicles

Before the development of more efficient extraction techniques and sensitive bioassays for endogenous CK's, only GA's and inhibitors were implicated in the control of dormancy and germination, particularly of seeds which required a chilling treatment to break dormancy (Wareing and Saunders, 1971). Ethylene can also overcome dormancy in some seed. Because these three PGRs (GA's, CK's and Ethylene) are found in seeds, it has been proposed that together they may control the release from dormancy (Roberts and Hooley. 1988). In general, there have been inconsistency in reports that applied PGR's are partially or completely ineffectual (Black, 1980/81). Black reasoned that these failures could have been due to (i) the use of wrong promoter (CK's or GA's) , (ii) the promoter failed to enter the seed and (iii) dormancy breaking was not susceptible to hormonal control.

(ii) Dry storage

The seed of many plant species, having completed their development on the parent plant, will not germinate under favourable conditions unless they have been stored for a period in dry storage. Crocker and Barton (1953) listed 42 species with this type of dormancy. However, in many cases, including the cereals, the dry after-ripening period can be negated or shortened if substituted by a short cold, wet stratification treatment. If dormancy is to be removed by stratification or light treatment, the seed must be partially or fully imbibed to be effective (Stokes, 1965). Under these conditions the seed is biochemically active and the block to germination is removed by a process that is apparently metabolic. On the other hand, Beldrok (1961) has shown that the duration of the dormant period in dry-stored wheat seeds is much reduced by exposing grains to desiccation. Since the loss

of this form of dormancy is favoured by dry conditions, there is some doubt in this case that the block to germination would be removed by a metabolic process. With regard to seeds after-ripening in dry storage, however, the tendency has been to regard the process as the expression of purely physical changes in the seed (Thomas, 1972).

Biochemical explanations of after-ripening are proposed by Simpson (1965) and Roberts (1969). The exact nature, however, of the after-ripening process that enables the seed to overcome the block to oxidative metabolism is not clear.

(iii) Elevated temperature

Warcup (1980) simulated the temperature enhancing effects of fire or sunlight on seeds buried in samples of forest soil by using a series of steam heatings. The lowest temperature treatment (55°C for 5 - 30 minutes), increased the amount of germination of seeds of some species, mainly members of *Juncaceae* and *Cyperaceae* . Families that produce hard seeds such as the *Leguminoseae, Geraniaceae and Convolvulaceae* had increased germination in soil which had been heated up to 60 or 70°C for 30 minutes. Beadle (1940) reported the role of fire in maintenance of the bush ecosystems in Australia, in which the heat of the fire brings about the breaking of seed dormancy. Bradbeer (1988) contended that it was likely that the dry storage or heat treatments damaged the seed coats of such seeds, so that both imbibition and germination became possible. The strophiole of *Acacia kempeana* was shown to lift and crack in response to heat (Hanna, 1984) allowing water entry into the seed. It is possible therefore that dry storage and heat may have a common

mechanism, in that they may effect organisation of membranes in the embryos of such seeds (Bradbeer, 1988)

(iv) Light

The subject of light controlled seed germination has been comprehensively reviewed by Evenari (1965), Wareing (1969), Taylorson and Hendricks (1977) and Karssen (1980/1981). Many of the effects of light on plant morphogenesis can be interpreted in terms of changes in a photoreceptive pigment or photochrome (Borthwick *et al* , 1952). The phytochrome hypothesis explained the previous observations of Flint and McAlister (1935, 1937) that light at a wavelength of approximately 650 nm promoted whereas that at 750 nm inhibited the germination of lettuce seed. This observation has formed the basis of most subsequent explanations of light promoted seed germination. There are three spectral regions active in the phytochrome system, blue (450 nm), red (650 nm) and far red (750 nm) light. Red light (RL) ends seed dormancy and promotes germination. Blue and Far Red light inhibit germination.

Kohler (1966) reported that increased GA levels could be detected in lettuce seeds 1 hour after irradiation with red light (RL) had commenced. Smolenska and Lewak (1971) showed that short exposure to white light caused a 3-fold increase in GA levels in isolated apple embryos. Continuous irradiation with white light increased endogenous GA in seeds of *Pinus sylvestris* (Kopcewicz, 1971). In addition, short periods of RL could also cause a rapid increase in GA in seeds of *Picea sitchensis* and *P. sylvestris* (Taylor and Wareing, 1979).

Increase in the level of endogenous CK's have also been reported following exposure to RL (Van Staden and Wareing, 1972; Van Staden,

1973; Taylor and Wareing, 1979) following the discovery that exogenous application of GA promoted dark germination of many light requiring seeds and that CK was effective in other species, it was assumed that the effect of light in releasing dormancy in such seeds was mediated through such hormonal increases.

Not all light requiring seeds, however, can be induced to germinate in the dark by treatment with exogenous GA's or CK's and in some seeds there appeared to be synergistic effects between RL and exogenous growth hormones (Wareing,1982). Synergistic reaction were also evident between RL and CK thus K only promoted germination of lettuce seeds if they were first exposed to a low level of RL. This indicated that a minimal level of P_{fr} must be present for K to be effective in promoting germination (Black, *et al.*, 1974). The above research indicates that , in some seeds the effect of RL in promoting germination are partly mediated by increases in endogenous GA's and/or CK's, but that P_{fr} must also be affecting other processes essential for germination. The effects of RL on endogenous hormone levels may be a necessary but not sufficient condition to enable germination to proceed (Wareing, 1982). This conclusion ties up with the evidence that phytochrome acts by controlling membrane permeability (Wareing, 1982). As such, it may affect compartmentalisation in the cell and conversion to P_{fr} may bring enzymes and substances together where before they were separated. The production of free GA may only be one of the processes inititiated in this way (Wareing, 1982). Moreover , RL may be controlling the release of free GA from inactive forms, rather than by *de novo* synthesis. Some evidence to support this hypothesis can be found from studies which observed rapid increases in GA level in etiolated

barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.) leaves in response to RL (Loveys and Wareing, 1971; Cooke and Saunders, 1975; Evans and Smith, 1976). Despite the very substantial literature, our understanding of the mechanism of action of phytochrome and consequently of the way in which light breaks seed dormancy remains, however, fragmentary (Bradbeer, 1988).

(v) Scarification

Both physical and mechanical constraints of the seed coverings will cause hardseededness in seed (Table I.1). Hardseededness is a term more appropriately used to describe seed which exhibits physical rather than mechanical dormancy (Gordon and Rowe, 1982). True hardseededness under natural conditions is removed gradually , mainly by the action of biological decay. The obvious dormancy-breaking mechanism involves damage to the seed coverings. Natural scarification can be brought about by various means eg. trampling by hoofed animals; partial digestion through the gut systems of rodents, birds, insects; degradation by fungi and soil microorganisms. If breakdown of the seed coats in these cases results from a slow physical and chemical breakdown either by oxidation and the absorbance of solar radiation, then dormancy-breaking may take years to achieve. In such cases, in a population of seeds, germination will be sporadic (Gordon and Rowe, 1982). To overcome such dormancy, and achieve rapid, synchronous germination, seeds may be manually scarified either by physical or chemical treatments which destroy coat integrity and allow the process of imbibition to proceed. On a large-scale, scarification involving abrasion, percussion or grinding has been mechanised (Bradbeer, 1988). Alternative procedures include treatment with concentrated sulphuric acid, normally for anything from 5 to 60

minutes (Bradbeer, 1988) or immersion in hot or even boiling water. Over exposure may damage embryos and the duration of the treatment must be determined experimentally for each seed lot (Blundell, 1973; Gordon and Rowe, 1982).

Physical dormancy as defined by Nikolaeva (1977) is maintained by a relatively thin seed coverings that became permeable as soon as seeds are subject to minor injury. However, seeds (*Crataegus spp., Carpinus spp.* Gordon and Rowe, 1982) showing mechanical dormancy normally have a tough, leathery or fibrous outer covering which is both thick and permeable. In these cases concentrated sulphuric acid not only scarifies the seed coat but also penetrates to the embryo. Therefore, the use of acid scarification treatments where hardseededness is not a problem is not recommended (Ellis *et al.*, 1985). In addition with regard to mechanical dormancy, scarification is even less reliable than acid scarification and hot water treatments are totally useless (Gordon and Rowe, 1982).

(vi) Treatment with chemicals

Several compounds have been applied to seeds in an effort to break dormancy. Many have been found to promote the germination of dormant seeds (Ellis *et al.*, 1985). Such chemical includes: hydrogen peroxide, ethyl alcohol, sodium hypochlorite, thiourea, mercaptoethanol, nitrates, nitrites, hydrogen ions (i.e. low pH), cyanide, azide, gibberellins, cytokinins and hydroxylamine.

Potassium nitrate (0.2 %) is customarily used to break dormancy in viability tests on many commercial seeds species. A top dressing of nitrate on young crops in spring is frequently followed by a great flush

of weed seedlings. In species of Japanese black pine and Scots pine *Pinus thunbergiana* and *P. sylvestris,* respectively, the use of potassium nitrate can replace the application of light as a condition for stimulating germination (Asakawa and Inokuma, 1961; Nyman, 1963). These chemicals affect the embryo, not the seed coat (Krugman *et al*..1974). However, some chemicals like organic solvents may effect the embryo directly by effecting inhibitor extraction or membrane modification (Bradbeer, 1988).

Seedcoat permeability and germination have long being known to increase by soaking seed in ethyl alcohol eg. *Acacia spp., Cercis spp.* (Barton, 1947), and by the use of xylene, ether, acetone, and chloroform (McKeever, 1937). Other chemicals such as citric acid, thiourea, and hydrogen peroxide can also stimulate germination in some species of dormant seeds (Ching and Parker, 1958; Hubbard, 1958; Frankland, 1961 and Cotrufo, 1962).

The success of CK's and GA's has been highlighted earlier (section I.2.2.1.4). Recent studies on exogenous application of Plant Growth Regulators (PGR's) have emerged in support of their effectiveness in alleviating dormancy in some woody plant seeds, for examples, application of GA on *Oldenlandia corymbosa* (Corbineau and Come, 1980/1981), *Rhus typhina* (Norton, 1985; 1986) or ethephon treatment (Joshi, Mishra and Gour, 1978; Norton, 1985; 1986). Gordon and Rowe (1981) studied the effects of GA_3 and $GA_{4/7}$ on seed germination of *Nothofagus obliqua* and *N. procera* . They found that a 24 h soak in GA_3 at 50 and 200 mg/l or $GA_{4/7}$ at 24 and 48 mg/l produced vitually 100 % germination of viable seeds which otherwise normally required a six week

pre-chilling treatment at 3-5°C to induce satisfactory germination in the nursery.

Studies to improve germination of serviceberry (*Amelanchier alnifolia*) which have an impermeable seedcoat and embryo dormancy, was carried out by Weber *et al.*, (1982). Its dormancy is normally overcome by cold stratification or autumn sowing in the Western United States. A combination of acid scarification for 30 minutes and a mixture of thiourea (300 mg/l) and benzyladenine (100 mM) as media moistening agents can also overcome dormancy in this species.

(vii) Dormancy breaking by fluctuating temperature

Fluctuating temperature can break dormancy in many seeds. Temperature in nature follows both seasonal and diurnal cycles; in the soil the same cycle also varies according to the depth of the soil (Russell, 1973). Diurnal temperature fluctuations in the uppermost few centimetres of soil, where most seeds germinate, are extreme, varying from as low as 5°C to as high as 35°C over 24 hrs. A dormant seed which germinates when exposed to diurnal fluctuations is responding to a system which senses its position in the soil profile. It has been found in *Rumex obtusifolius* L. and *R. crispus* L.that limits of the cycle are separated by about 15°C and the most favourable temperature are around 15°C and 35°C, although other temperature patterns may prevail (Attims and Côme, 1978; Totterdell and Roberts, 1980).

Alternating temperature, usually in the range of 10 to 40°C have long been known to affect markedly the breaking of dormancy (Thompson, 1974). The period of a cycle is usually a day, with the time at the lower temperature longer than at the higher temperature. Effects of a

single shift to higher temperature, however, can sometimes be attained in minutes (Taylorson and Hendricks, 1972). The effect of a temperature alternation interacts strongly with phytochrome, often synergistically. Effects of the higher temperature are associated with changes in the membrane permeability as displayed by leakage of endogenous constituents from the seeds (Hendricks and Taylorson, 1976). If the high temperature is maintained for too long, the loss of materials from the seed is deleterious to germination. High temperature will result in the leakage of organelle membranes and enhances metabolic activity over that at lower temperature (Taylorson and Hendricks, 1977).

The effect of fluctuating temperatures on the germination of 112 species of herbaceous plants in the Sheffield area of England were investigated (Thompson and Grime ,1979), which in a previous study had failed to germinate to a higher percentage in the light or which responded only to exceptionally high (> 25°C) constant temperatures. The germination experiments were carried out on a thermogradient bar in which part of the apparatus was held at a constant 22°C with the remainder subjected to a depression of 1 - 12°C for 6 hrs in every 24 hr. Forty-six of the species examined were found to have their germination stimulated by temperature fluctuation in the light. In another study (Grime *et al.*, 1981) it was found in the 16 species studied, that germination in the light was found to be dependent upon exposure to diurnal fluctuations in temperature.

Plants as have been alluded to above have become adapted to their own niche and environment and their seeds have different warm and cold requirements for synchronizing successful germination in the most efficient time possible. *F. angustifolia* and *F. excelsior* are

stimulated to germinate by a few weeks at temperatures below 10°C and a protracted cold treatment of 1 - 3°C respectively. One reason for the differential response is perhaps that the former are naturally found in warmer temperate zones whereas the latter are endemic to colder climates (Gordon and Rowe, 1982). Another example is also found in *Liriodendron tulipifera* , a species with an extensive range of natural habitats and different land races of which may respond differentially depending on their geographic location (Barnet and Farmer, 1973).

The application of alternating temperature regimes as prescribed by the International Seed Testing Association (ISTA) are very effective in breaking dormancy. In practice it is sufficient if the warm temperature is more or less constant within the range of 20 to 25°C, while the cold is within a range from 1 - 5°C. However, during temperature treatments the seeds must be kept moist and adequately aerated (Gordon and Rowe, 1982). For this, a moist compost mixture is superior to moist sand during warm treatment since the mixture reduces the degree of compaction and increases both the moisture and oxygen retaining qualities of the medium.

2.2.1.5 Earlier studies of *Sorbus aucuparia* seeds.

There were only three reports found in the literature that specifically deals on the seed problems of this species. This lack of knowledge about the species clearly justifies a re-evaluation of the earlier results and new approaches should be attempted in order to address the problem of seed dormancy in this species.

I. The initial and most extensive studies carried out on *Sorbus aucuparia L.* was made by Flemion (1931). Her findings are summarized as follows:

(a) Seeds are dormant and require an after-ripening by prechilling for between two and four months. Prechilling at 1°C was better than at 5°C producing a percentage germination of 94 % compared with 39 % respectively.

(b) Granulated peat moss (pH = 4) was found to be superior as a stratification medium compared to sand, manure or peat moss with a pH more alkaline or more acid than 4.

(c) Attempts to break seed dormancy by treatment with various chemicals failed. The failure was mainly attributed to the impermeability of the seedcoats to the chemical tested. The chemicals were not infused into the embryos. They were; ethylene chlorohydrin, thiourea, thiocynates, potassium cyanide, urea, saponin, vanadium compounds, triethanolamine, alcohol, glycerine, hydrogen peroxide, adrenaline and cyanates.

(d) The seeds were partially after-ripened when stored dry for 6 months at room temperature so that the subsequent after-ripening and germination period required at 1°C was shortened.

(e) Seeds stored for two years in sealed and unsealed containers under vacuum, at temperatures ranging from -8°C to unspecified room temperature were just as viable as freshly harvested seeds. The viability was rapidly lost when stored at 25°C whether sealed or unsealed.

(f) When partially or entirely after-ripened seeds were stored dry at high (10°, 15° and 20°C) or low (1°C) temperatures or when partially after-ripened seeds were kept moist at higher temperatures (10°, 15° or 20°C), a secondary dormancy developed so that the seeds required a second prechilling period which was found to be longer than the original stratification period.

(g) The excised embryos after-ripened in 6 weeks when exposed to 1°C temperature stratification, thus showing that the seedcoat played a role in the dormancy of these seeds.

(h) Excised dormant embryos were placed on moist filter paper and exposed to light, the cotyledon which was in contact with the wet medium became enlarged and turned green in colour, whereas those that were kept in the dark became yellow. Those that turned green did not produce seedlings, however they showed some germination when their water and oxygen supply were increased by aerating the water but growth was very much slower than that of after-ripened embryos.

(i) Extending the findings in (h) above, Flemion (1937), developed a rapid viability test of various dormant seeds by observing the behaviour of excised embryos when placed on moist filter paper in petri-dishes at room temperature.

II. The first attempt to study growth inhibitors in *Sorbus aucuparia* seeds were made by Barton and Solt (1949). Extracts were made of dormant and non-dormant seeds and their effects on the growth of wheat roots in solution were tested. They were able to prove that dormant rowan seeds contained materials which inhibited the growth of wheat roots.

III. The third study on rowan seeds was performed by Hilton *et al.*, (1965). It was aimed to unravel the nature of the inherent dormancy in three rosaceous species which included rowan. Their work is summarized as follows:

(a) Quick testing of viability of seeds followed the method described by Flemion (1931) i.e. the cotyledon greening test and the tetrazolium test (TTZ). The cotyledon greening test required 6 to 7 days for an estimate to be made. They found a high degree of correlation between the two techniques but found that the TTZ test was a quicker and more satisfactory method.

(b) They found a positive relationship between germination and (i) low (2°C) after-ripening temperature, (ii) increasing duration of after-ripening period, and (iii) scarification with sulphuric acid. An after-ripening temperature at 2°C was reported to be superior to either 6 or 10°C. Treatment with concentrated sulphuric acid for 10 minutes was better than either treatment with concentrated NaOH for 20 minutes or the mechanical abrasion by rubbing with sandpaper to an exposed part of the embryo. The combination of scarification and prechilling at 2°C for 3 months with sulphuric acid produced an average of 75 % germination, NaOH produced 64 % germination, the control seeds exhibited 58 % germination and by abrasion only gave 10 % germination. The low percentage of germination obtained by abrasion may have been due to an inward leaching of growth inhibitors from seed coat tissues, enforcing a longer resting phase to the seeds.

(c) They also determined the presence or absence of growth inhibitors by means of chromatographic extracts from seeds. Eluates from R_f regions 0.8 to 0.9 were found to have had a marked inhibitory effect upon the elongation of Genesee wheat coleoptile sections, the primary root elongation of germinating vegetable seeds and the growth of *Sorbus* seedlings. This indicated the presence in the seeds of compounds that strongly inhibited growth.

IV. Zentch (1970) investigated the stratification of *Sorbus aucuparia* seeds interspersed with warm phases for up to six months. He concluded that a warm-followed-by cold stratification was not superior over the cold one, as it neither increased the germination percentage, nor shortened the necessary cold treatment.

V. Gordon and Rowe (1982) recommended for *Sorbus aucuparia* seeds that an effective pre-sowing treatment to break dormancy was a two week warm followed by a cold stratification. All authors prior to this , which included ISTA (1985), believed that a cold moist stratification was the most efficient treatment to break dormancy in this species.

VI. The most recent report was of Lenartowicz (1988) which studied the effect of the initial warm phase (0 - 32 weeks), as well as the temperature of the warm (15 - 25°C) and cold (1 - 5°C) stratification phases, on germination of *Sorbus aucuparia* seeds. His accounts proved the effectiveness of the warm followed by cold stratification for inducing a synchronized germination of rowan seeds, which had not been obtained in earlier studies. At the same time, however, the time of the chilling period required was prolonged.

2.3 Vegetative propagation by tissue culture in forestry

2.3.1 Introduction

Most mass planting of forest trees are made from seedlings.The seedlings being produced from seed. The seed is usually obtained from crosses between selected clones from breeding programmes (George and Sherrington, 1984). The selected clones have usually been established in nursery seed orchards by grafting onto rootstocks. For the maintenance of superior genotypes that would be lost in meiotic segregation and recombination, elite trees are reproduced vegetatively by traditional means.

Traditional vegetative propagation (cloning) of trees by cutting and grafting is a useful tool in conventional tree improvement (Libby, 1986). Preferred genotypes can be replicated and propagated. The validity and importance of clonal propagation for woody species is irrefutable. The popular practice of vegetative propagation is through rooting of cuttings, but in many species, this method has proved difficult. An alternative choice of propagation for difficult-to-root species is lacking.

The major problem with rooted cuttings is the rapid loss in rooting capacity with increasing age of the elite parent tree (Rao and Lee, 1982; Abdullah, 1987), thus rendering the selection on the basis of their good past performance unsuccessful. Moreover, with this traditional method of propagation the speed of rooting, root length and number, survival and growth, all decline when the parent plant is more than 10 years of age (Girouard, 1974). Treatments that promote rooting in some may have no effect on the rooting response of other species or

cultivars within a species. Nonetheless, cutting and grafting methods which have been practiced in agriculture, horticulture and forestry for centuries, without doubt, will remain an important aspect of propagation of such plants. More research is needed to solve the problem of rooting in cutting materials and clearer scientific understanding of the physiological causes responsible for differences have to be unveiled. Also, clonal propagation by cutting and grafting have other limitations especially when large quantities of propagules are required. With the advance in technology of propagation, tissue culture techniques offer an alternative for the mass propagation of woody plants particularly the ornamentals and hardwood tree species. The technique has been succesfully applied to many of the tree species, especially in case of certain gymnosperms (Rao and Lee, 1982). Although there is a general consensus that, at present, the economic cost is a serious handicap for practical acceptance (Sommer and Caldas, 1981; Debergh, 1988). But this technology could be trimmed of unimportant details and simplified to make it speedy and less expensive. *In-vitro* selection offers great potential for improving forest trees. The successful application of this technology to many fruit trees for the production of clonal rootstocks (eg. in peaches, apples, pears and so on) may one day be extended more meaningfully in the reforestation programmes of the hardwood tree species. The main tissue culture propagation barrier thus far has been the long life cycle of the tree species and difficulties in applying current biotechnologies to mature and proven provenances (Durzan, 1985).

Tissue culture presently has reached a level of sophistication where its adaptation to large scale industrial use has become a reality

in some areas of agriculture and horticulture. However, its application in forestry is still in its infancy (Bonga and Durzan, 1982) and efforts must be expanded to realise the full potential of this fast developing technology (Karnosky, 1981; Durzan, 1985; Thorpse, 1985: Yeoman, 1986).

2.3.1.1 Historical perspective

The emergence of tissue culture of trees or woody plants is not a recent happening, in fact trees were among the first plants to be cultured. Haberlandt (1902) was the first to envisage the concept of the totipotency of the plant cell, which is the ability to regenerate a whole organism from a single cell. This concept opened up a completely new frontier in the art of propagating , aseptically and heterotrophically. Three decades were required to bring this methodology into practice. It was Gautheret (1934) who first cultured the cambial tissues of *Abies pectinata* (Fir), *Acer pseudoplatanus* (Sycamore), *Alnus glutinosa* (Alder), *Fagus silvatica* (Beech), *Fraxinus excelsior* (Ash), *Pinus maritima* (Corsican Pine), *Populus nigra* (Black Poplar), *Quercus robur* (Pedunculate Oak), *Salix caprea* (Sallow) and *Ulmus campestre* (Scotch Elm). Tissues from *Acer* (Maples), *Populus* (Poplars), *Salix* (Willows) and *Ulmus* (Elms) developed best. The embryos of *Picea canadensis* (Spruce), *Pinus resinosa* (Red Pine), *Pseudotsuga menziesii* (Douglas Fir), *Thuja occidentalis* (Northern White Cedar) and *Tsuga canadensis* (Eastern Hemlock) were succesfully cultured into normal-appearing seedlings in culture by La Rue (1936).

Four years later, Gautheret (1940a, 1940b) reported the growth of buds obtained from cambial tissues of *U. campestre* (Scotch Elm) cultured *in vitro*. Morel (1948) reported the culturing of tissues from *Crataegus*

monogyna (Common Hawthorn), *Pyrus communis* (Common Pear) and *Syringa vulgaris* (Lilac) and their use in plant pathology studies. The first regularly subcultured angiosperm tree callus cultures were those from *S.caprea* (Sallow) (Gautheret, 1948), *S. vulgaris* (Lilac) (Morel 1948) and *Castanea vesca* (Chestnut)(Jacquiot, 1950) with some calli forming a few small shoots or roots (Jacquiot, 1966). It was the work of Ernest Ball who reported the differentiation of buds in somatic tissue cultures of *Sequioa sempervirens* (Coast Redwood) (Ball, 1950), which marked the start of an intensive interest in the *in vitro* culture of conifers. The growth of apices of *S. vulgaris* (Lilac) in culture were later attempted by the work of Wetmore(1954). Using a Knop's solution supplemented with sucrose, the apices could be grown only if planted upon a nurse culture. However, when 15 % autoclaved coconut milk and 0.4 ppm casein hydrolysate were added to the nutrient medium, the apices could be induced to produced shoots which later rooted.

The first indication that buds from gymnosperm tissue culture could form shoots were reported about twenty-four years ago through the work of Konar and Oberoi (1965). They discovered that the cotyledons of *Biota orientalis* embryos in culture developed structures they named 'embryoids'. Transplanting them encouraged the formation of shoot but not roots. This species has been reinvestigated (Thomas et al., 1977), and the adventitious organs found were not embryoids but rather buds i.e., no structure resembling a radicle was found.

The pioneering work of these men represent the beginning of tree tissue culture and its advances was enhanced by the development of better media by; Reinert and White (1956), Murashige and Skoog (1962),Riser and White (1964) and Brown and Lawrence (1968).

2.3.1.2 The rationale for the development of forest tree tissue culture

The shortage of wood products has been predicted in many reports, world wide demand for industrial raw material is expected to increase by 3 to 4 % annually, and is likely to continue to rise until the year 2000 (Stockman, 1980). Wood use increased from about 730 million cubic meters in 1960 to about 1233 in 1980. It is forecast to surpass 1800 million cubic metres by the year 2000 (FAO, 1982). Any intensive reforestation programme, requires a vast quantity of planting stocks (Rao and Lee, 1982). Present day tree improvement practices offer only limited possibilities to achieve such goals. Novel techniques that can be used, in addition, to the traditional ones are required. Tissue culture which has developed since 1930s shows promise and its use in forestry should be considered.

A clear distinction between forest and non-forest (eg. fruit and ornamental trees) is difficult, and since the problems of propagation and genetic improvement are largely the same in both groups.

Tissue culture or micropropagation is only meaningful when elite and selected mother plants are used as a source of explants. When juvenile materials (seeds, embryos and seedlings) were used as starting material, one has no (or not enough) information about the intrinsic qualities of the plant, and it is impossible to evaluate the trueness-to-type of the offspring (Debergh, 1987b). In woody species this selection is most often only possible at an advanced stage in plant life. Therefore, whenever vegetative propagation of mature explants is economically possible it is generally preferred to sexual propagation or

propagation from juveniles because genotypic characteristics are better maintained (Thulin and Faulds, 1968; Murashige, 1974). Through tissue culture methods as proven for many commercial fruit trees such as propagation of apples, it is possible to propagate rapidly, trees with outstanding and requested genetical properties; for instance rapid growth, large production of wood volume, increased wood quality, resistance to air pollution and resistance to disease. Simultaneously the method can be used for rapid propagation of tree species where the seed production is low, such as those climax species of the tropical rainforest . Therefore, *in-vitro* propagation systems offer great potential for improving trees. The advantages of clonal multiplication of plant species using tissues culture technology are many, and because of that, it is becoming established as a commercial method for the production of many species of forest trees and non forest trees. According to McCown (1982) more than six genera of deciduous tree species are being commercially propagated by shoot tip culture techniques and production rates of 15,000 to 50,000 plantlets per square metre of culture space per year can be achieved.

2.3.2 Micropropagation of broadleaved trees (Angiosperms)

Micropropagation of fruit and tree species has been reviewed by Bonga (1977b), Button and Kochba (1977), Abbott (1978), Sharp *et al* (1979), Skirvin (1981), Sommer and Caldas (1981), Mott (1981), Bonga and Durzan (1982), Fujuwara (1982), Lane (1982), Rao (1982), George and Sherrington (1984), Bajaj (1985), Henke *et al* (1985), Rao and Lee (1986).

The term 'tissue culture' was first coined in the days when the technique was restricted to the culture of pieces of tissues. It has now

progressed over the years, such that the term has become a misnomer. It now covers a wide range of explants not only tissue segments but also free cells, protoplasts, organs (e.g. shoots, roots ...etc.) and embryos are cultured (Bonga and Durzan, 1982). Plant tissue culture is the *in vitro* cultivation of plant pieces or segments, tissue or cells under aseptic conditions in or on a nutrient medium under controlled conditions of light and temperature. The sterile culture medium normally consists of inorganic macro and micronutrients and vitamins, and may include other organic nutrients such as amino acids and organic acids (Murashige and Skoog, 1962; Gamborg and Shyluk, 1981) and growth regulators. These provide the essential chemicals required for growth.

2.3.2.1 The techniques

For convenience the techniques of tissue culture can be classified into five classes, namely, callus culture, cell culture,organ culture, meristem culture and morphogenesis and lastly protoplast culture (Gamborg and Shyluk, 1981). For the purposes of *in vitro* plant production or micropropagation, some of the techniques have more relevance than others.

Rapid asexual multiplication of viable propagules *via* tissue culture can be achieved through, (1) shoot proliferation of existing meristems from apical and axillary buds, (2) multiplication by adventitious shoot production either directly from organ explants or from callus, and (3) somatic cell embryogenesis. However, *in vitro* culture of (4) seed embryos (Wilkins and Dodds, 1983) is a well-established procedure of regenerating plantlets for a variety of reasons.

Production of plants from system (1) and (2) requires rooting of individual shoots to produce complete plants. For purposes of clonal propagation system (1) is preferred, since meristem or shoot derived cultures are generally believed to be genetically stable (Murashige, 1974). This may be due to the capacity of these meristems to undergo continuous cell divisions and to regulate precisely the sequential events of DNA synthesis and mitosis.

2.3.2.1.1 **Multiplication of existing meristems**

Axillary and apical shoots contained quiescent or active meristems and are capable of growth into shoots identical to the mother plant (Tisserat, 1985 and Hussey, 1986). Micropropagation techniques using these meristems are popular with a high degree of success in producing uniform clones.

Many workers have described in detail the procedure for regeneration of tree plantlets from a tissue culture system. The principle stages usually followed are those put forward by Murashige (1974). According to his guidelines four essential stages are considered. 1. Establishment of the aseptic culture. 2. Shoot multiplication. 3. Rooting of developed shoots and, 4. Hardening of plantlets for establishment in soil, in a 'normal' environment.

(i) Establishment of the aseptic culture

The performance of this stage is considered a success when an acceptable proportion of explants have survived, and grown *in vitro* media without contamination by microorganisms or fungi.

Explants either taken from mother plants growing in the external environment or from seed embryos are contaminated with microorganisms. These explants must first be sterilised. Commonly used surface sterilants include sodium and calcium hypochlorite, mercuric chloride, hydrogen peroxide, and other commercial bleaches. The first two reagents are preferred by most workers, since mercuric chloride is considered to be highly toxic to most plant tissues. The type and concentration of sterilant to be used and exposure time must be decided upon empirically. Chalupa (1981) successfully micropropagated thirteen broadleaved trees including *P. tremula* (aspen), *S. alba* (white willow), *S. viminalis* (common osier), *Betula verrucosa* (silver birch), *B.pubescens* (downy birch), *Carpinus betulus* (hornbeam), *A. glutinosa* (common alder), *A. incana* (grey or speckled alder), *Q. robur* (pendunculate oak), *T. cordata* (little-leaved linden), *S. aucuparia* (rowan), *Juglans regia* (common walnut) and *Robinia pseudoacacia* (false *Acacia*) . He used 0.1 - 0.3 % of mercuric chloride solution for 15 - 30 minutes to surface sterilize all the explants. The exposure time to sterilisation was varied according to the season of the year and maturation of the exlants used. Sommer and Caldas (1981) used 5 % sodium hypochlorite solution to surface sterilize explants such as tree seeds and buds. For softer tissues, a dilution to a lower strength may be needed but anything below 0.5 % may prove ineffective in killing the majority of microorganisms. It is important that the plant surface be properly wetted and Tween 20 is normally added to the sterilizing solution . Sterilants must be removed by thoroughly rinsing with several changes of sterile distilled water.

(ii) Shoot multiplication

The main aim of this stage is to obtain an optimum number of viable and useful multiple shoots or propagules. This can be achieved either by microcuttings or by encouraging the release of axillary buds.

It is usually observed that the shoot tip when grown in a medium devoid of plant growth regulators, tends to develop a single long shoot with a strong apical dominance.By microcutting the nodal segments, the number of shoots could be increased by further culturing. The propagation rate by microcutting is rather limited.

A higher multiplication rate of shoots is normally achieved by incorporating plant growth regulators (usually CK with a low level of an auxin) into the culture medium which reduces the effect of apical dominance and promotes the growth of lateral shoots. The multiplication rates achieved depends upon the species, whether or not the shoots are juvenile or mature and the individual genotype of the tree. Other equally important factors that affect the proliferation rates are the chemical composition of culture medium, the physiological status of explants and stock materials, the size of explants and the environmental conditions of the stock materials (Hu and Wang, 1983).

The CK usually used with trees is the synthetic compound 6-benzylaminopurine (BAP) which stimulates the shoot explants to grow and enhances branching of lateral buds from leaf axils forming shoot clusters. Shoots from the cluster can be further subcultured onto fresh medium to produce further shoot clusters. An important feature of the response to BAP is the progressive increase in the rate of axillary shoot proliferation with monthly subculture (Hutchinson, 1984) as

observed in apples. Although the shoots of some apple cultivars proliferate rapidly on a medium with CK as the only growth regulator (Lane, 1978), the addition of a low concentration of an auxin, usually indole-3-butyric acid (IBA), may enhance shoot proliferation and elongation to some extent (Zimmerman and Broome, 1980), and there have been similar benefits with cherry and plum (Jones, 1983).

For *S. aucuparia* (rowan) (Chalupa, 1981), the sources of explants used were shoot tips and nodal segments from actively growing two year old seedlings. A Murashige and Skoog (MS) medium supplemented with cytokinin (BAP) stimulated rapid growth of multiple shoots from axillary buds. A high multiplication rate was achieved on a medium containing a higher concentration of BAP (0.6 mg/l) and a low concentration of auxin (IBA 0.05 mg/l).

Similarly, for *P. tremula* (aspen), a higher concentration of BAP (0.6 mg/l) and low concentration of auxin (0.05 mg/l of NAA (- naphthalene acetic acid) or IBA) stimulated growth of numerous axillary shoots and often adventitious shoots were also produced.

(iii) Rooting of developed shoots

The *in vitro* rooting of tree plantlets of angiosperms has been relatively easy, whereas gymnosperms have presented more problems (Sommer and Caldas, 1981). A modified Morel's medium (Start and Cumming, 1976) with 0.1 - 10 mg/l IBA has given satisfactory results with the African violet (*Saintpaulia lonantha*). However, Hu and Wang (1983) found that rooting of excised shoots from *in vitro* cultures can be very difficult to achieve in woody species. Several different methods have been devised by various workers to induce rooting of aseptically

produced shoots. Most of these methods consist of a two step process of, firstly, root initiation by exposure to an auxin, followed by transfer of the shoot to an auxin free medium to allow root elongation.

Others have reported that for root formation on developed shoots, the auxin concentration in the medium must be higher than the CK concentration . The presence of an auxin is essential for the induction of root initials or primordia. Rejuvenation of the shoot cultures as manifested by an increase in the rate of shoot proliferation with subculture on a medium with CK, is accompanied by an increase in the capacity of the shoots to initiate adventitious roots. Half-strength MS is generally better than full strength . Sucrose at 1% is generally better than at 2 or 3 %. A combination of these factors must reduce callusing at the base of the shoots, otherwise the callus may grow faster than the root primordia and eventually inhibit root growth.

For *S. aucuparia* (Chalupa, 1981), rooting of shoots was achieved on the low salt, low sucrose medium (0.5 - 1 %) and low concentration of auxins (0.3 mg/l NAA and 0.3 mg/l of IBA). Eighty-five to 100 percent of shoots rooted within 2 - 3 weeks. Similarly, for *P. tremula* (aspen), (Chalupa, 1981) root initiation was promoted on a MS medium containing low concentration of sucrose (1 %) and low concentrations of auxins (NAA 0.2 mg/l and 0.1 mg/l of IBA).

Excised shoots from culture could also be rooted *in vivo* as reported by McCown and Amos (1979) where 100% rooting was obtained with *B. platyphylla* (Birch) shoots. The shoots were transferred into rooting chambers containing a 1:1 mixture of peat and perlite at 30 to 35°C and humidity of 80 %.

(iv) Hardening off of rooted shoots for transfer to soil

Once a satisfactory root system has formed, the plantlets may be transplanted to sterile compost in a glasshouse or growth chamber. Plantlets are usually delicate at this stage and very sensitive to dessication so that a plastic propagator or mist application is necessary in order to maintain a high humidity. The plantlet needs to be acclimatized from very humid surroundings to one with varying and lower humidity. Adjustment needs to be gradual and this can be accomplished by removing the cover over the plantlets for intervals of increasing length over several days. Some shading from high light intensity, may help to prevent leaf scorch. This shading should also be gradually removed.Beside dessicatien another important factor that should be considered in the transplanting process is infection by fungi. Care must be taken in removing traces of agar for they provide a good substrate for pathogen growth. Normally potting media are sterilized to eliminate infection problems.

As progress in micropropagation techniques was made new methodology for production of woody plants developed rapidly. It became evident that the cost of production could be much reduced by simplifying the rooting stage from an *in vitro* step to an *ex vivo* one. Various techniques for direct *in vivo* rooting of micropropagated shoots were developed. It is already used far more extensively than would be indicated by the scientific literature, because most, if not all commercial laboratories develop their own techniques to improve results and cut costs (Zimmerman, 1988).

2.3.2.1.2 **Multiplication of plantlets through callus culture**

Callus culture is basically a system of growing many undifferentiated cells which retain the capacity to regenerate new plants. The culture arises from the disorganised proliferation of cells from explants of plant organs generated on an agar medium containing a combination of plant hormones and other chemicals the concentrations of which are dependent to a large extent on the species being cultured.

Regeneration of broadleaved trees from callus has been reported in *Populus, Quercus, Eucalyptus* and *Ulmus* species (George and Sherrington (1984). Various explants have been used for callus initiation and cambial tissue appears to be particularly productive.

For a system of micropropagation based on callus cultures, the sequence of procedural steps is essentially similar as shoot culture, except that initially a rapidly growing callus culture is normally established from the primary plant source. The hormone conditions are then changed so as to induce organogenesis (shoot formation or embryogenesis) within the callus tissue. Under the right conditions, shoots, roots or even the whole plantlets may be regenerated. Shoots produced may be excised and placed onto a root initiation medium as described previously, or embryo's may be excised and transferred to fresh medium for growth into plantlets. Juvenile tissues are generally most likely to form callus, and have been obtained from seedlings, buds, root tips or developing embryos such as fruits, floral parts, tubers, bulbs and so on (Gamborg and Shyluk, 1981).

The vegetative propagation of plants through callus culture has become an important tool to propagate a larger number of plant species.

Rapid multiplication of callus generally takes place when the tissues are grown in liquid suspension culture.

Plantlets can also be induced from protoplast derived calli. The possibility of using protoplasts for tree improvement has stimulated much interest because of success with herbaceous species. According to Thorpe (1985) the use of protoplasts allows for the blending of nuclear as well as cytoplasmic characters, thus raising the possibility of new genetic combinations by asexual means. Work with tree species has lagged behind but some success has nevertheless been achieved with tree species. There are few reports of protoplast culture of tree genera. *Citrus* (Vardi et al., 1975) and *Alnus* (Hughtinen et al., 1982/1983) are the only tree genera for which regeneration to callus or plants has been described (Jörgensen and Binding, 1984). Jörgensen and Binding (1984) were able to generate callus from protoplasts isolated from shoot tips of *S. aucuparia* (rowan) which may be taken as a promising advance to plant regeneration in this species. The protoplasts were cultivated in original and modified V-KM media (Binding and Nehls, 1977) at densities of 105 per ml, a temperature of 25 ± 1°C, and 800 lux of cool white fluorescent light. Within six days of culture, 3 % of the protoplasts showed mitotic activity. After 30 days, the cell clusters were transferred to medium B5C (medium B5 with 5 % liquid coconut endosperm and 2.5 uM BA; Binding et al., 1982) resulting in vigorous callus proliferation. However, no organogenesis has been obtained.

2.3.2.1.3 Embryo culture

(i) Background to embryo culture

One of the oldest applications of tissue culture in plant breeding is embryo culture (Bonga, 1977). Maheshawari and Rangaswamy (1965) have reviewed the extensive literature on embryo cultures of interspecific crosses in fruit trees. Trees have been raised from hybrid embryos of plum, apple, peach and pear. Raghavan (1977) reviewed the possible practical applications of the techniques directly related to the isolation and culture of embryos. Embryo culture has also shortened the breeding cycle and advanced germination of hybrid and nonhybrid embryos of apple, almond, plum, peach and cherry that are normally slow to germinate. Oil palm embryos often take several years to germinate under natural conditions, but their embryos, if excised and cultured *in vitro*, will germinate within a few days (Bouvinet and Rabëchault, 1965). Embryos and immature ovules of *Hevea brasiliensis* (rubber) have been cultured to obtain improved germination (Muzik, 1956). Nagai (1974) screened virus resistant papaya (*Carica papaya*) by raising plantlets induced from embryo culture *in vitro*.

Progress in embryo culture techniques can be used to 'rescue' and then culture hybrid crosses that would otherwise abort due to incompatibilities with the special nutritive tissue of the endosperm in angiosperms, or the female gametophyte of gymnosperms. In the tropical rainforest of South East Asia the gigantic dipterocarps flower only once every five to 15 years, however, some individual trees will flower in the years in between. But, it is almost impossible to get a constant supply of the seed for the planting stock. Embryo culture technology could supplement the shortage of seedlings by multiplication of plant-

lets *in vitro*.

In some types of seeds, which germinate only after a period of storage, dormancy can be overcome by excision and culture of embryos, and treatments generally used in dormancy breaking in other seeds are ineffective. In still other types of seed , whose dormancy is broken by specific light or temperature treatment, embryo culture has helped to localize the endogenous promoters or inhibitors of germination which presumably maintain the seeds in the nondormant or dormant state.

(ii) The techniques of embryo culture

Aseptic procedures are followed in the excision of the embryos. Most often surface sterilisation of the embryos is not necessary, since the embryos are protected by the seedcoats from contamination. For hard-coated seeds, isolation procedures begins by soaking in water for a few hours to a few days so that the embryos can be removed easily. Seeds are generally surface sterilized before and after soaking.

Aseptically excised embryos could be placed into a medium and this formed the simplest technique that can be used with seed. The most critical aspect of embryo culture is the choice of the medium and the necessary supplements to sustain continued growth of the embryos. The nutrient medium used to culture the embryos is species dependent, and are as varied as the number of species studied. It appears that the younger the embryos, the more complex is its nutrient requirement (Raghavan, 1977) whereby additional vitamins, amino acids, growth hormones and natural endosperm extracts like coconut milk has to be added as well as an inorganic salt and sucrose as a carbon source. A

mature embryo can be grown in just the presence of the latter two nutrients. Proembryos are normally embedded in the ovular sap and as such they were under considerable osmotic pressure, the presence of an osmoticum such as mannitol is recommended (Raghavan,1977). After the embryos have grown into plantlets *in vitro*, they are removed from the initial medium and hardened off in sterilized soil or vermiculite.

2.3.2.2 Factors affecting morphogenesis and proliferation

The factors to be considered when microculturing plant tissues in vitro can be grouped into five broad categories: (1) species and genotype (2) the physiological status of the explants and donor plants 3)composition of media i.e. minerals, hormones and other organics, supporting agents (Murashige and Skoog, 1962 and Gamborg and Shyluk, 1968) and 4) the choice of suitable surface sterilization techniques (Yeoman and Macleod, 1977) and 5) the environment such as light, temperature, gases, and vessels.

2.3.2.2.1 Species and genotype

Theoretically all plant materials can be cultured if the appropriate culture medium, correct hormonal balance and suitable environmental conditions are met. It is apparent that the success of any micropropagation techniques *in vitro* is influenced by the species and genotype than by any other single factor.

2.3.2.2.2 Physiological status of the explants and donor plants

The success of obtaining a response *in vitro* has been shown to be influenced by the growth conditions of the donor plant, which in turn presumably affects the physiological status of the tissues from which

the explants are derived (Hussey, 1986). Use of juvenile tissues as explants has been more successful compared to the relatively recalcitrant behaviour of the mature material in culture. The success of tissue culture diminishes with increasing age of the donor tree from which explants were derived , even though in some cases, juvenile characteristics are apparently maintained (Cheng, 1975).

2.3.2.2.3 **Adequate cultural environment**

With the advancement in the knowledge of the techniques of tissue culture a better picture has been obtained into the influence of culture medium and of the culture atmosphere on the efficiency of a micropropagation system.

(i) **Composition of culture media**

The success of a micropropagation system is largely due to a better understanding of the nutritional requirements of explants (Murashige, 1974). Many different media compositions, containing various concentrations of components, have been proposed as being suitable for tree tissue culture. However, these media all have in common a balanced and defined mineral salt composition together with the essential vitamins, a carbohydrate source and usually a CK or combination of growth regulators. The choice of a culture medium for any particular application appears to be largely a case of experimenting with several of the most commonly used media which have been employed by past workers for similar crop species. Once a medium has been found that allows satisfactory growth, it is then possible to make selective refinements to individuals media constituents in order to optimize culture growth.

(a) Mineral Salts (Inorganic)

The inorganic nutrients essential in plant cell culture are those required by a normal plant. Those required in millimolar quantities are Na, K, Ca, S, P and Mg . These elements are termed macro-elements and the optimum concentration of each nutrient for achieving maximum growth rates varies considerably.

The essential nutrients required in micromolar concentrations include Fe, Mn, Zn, B, Cu, and Mo. Addition of Co may be important but in several media it can be omitted. These second group of micromolar concentration are termed micro-elements.

The growth and morphogenesis of tissues is markedly influenced by the availability of nitrogen salts, and normally these are supplied by the addition of nitrate or ammonium ions alone or in combination (Murashige and Skoog, 1962; Schenk and Hildebrant, 1972; Gamborg and Shyluk, 1981).

McCown and Sellmer (1987) reported through his literature search that in general (a) a high ionic strength has an inhibitory effect on growth of many woody species , (b) When a low salt formulation does not support adequate growth of shoots, a marked improvement can be achieved by the addition of ammonium nitrate, (c) Ca deficiency in the tissues will result in the necrosis of the apex or shoot tip, this may in turn be due to (i) high humidity in the container and/or (ii) Ca not being mobilised in plant tissues in culture, and (d) cultures that look unhealthy probably leading to death is often attributed to high concentrations of chloride in the system.

Hussey (1986) mentioned that rooting of shoots is improved in many woody species and herbaceous species by lowering the concentration of macro salts to a half or less.

(b) Sugar as carbon energy source

A suitable carbon source for energy is necessary in the culture medium and the most commonly supplied is sucrose or glucose at concentrations 2 - 3 % (w/v) (Gamborg and Shyluk, 1981). However, Hussey (1986) recommended for good rooting of shoots that the concentration of sucrose be lowered to 0.5 or 1 %. This carbon source provides energy for active cell metabolism and is involved in cell division and tracheid formation (Minocha and Halperin (1974). Other carbohydrates have been tested, but all are reported inferior to sucrose and glucose. Sorbitol, however, has been used for cells of apple and other Rosaceous species. The presence of m-inositol is not an absolute requirement but it has been reported to improve cell growth when around 10 % is added to the medium.

(c) Plant Growth Regulators (PGR's)

Among the factors that influence organogenesis in tissue culture, the most important single component seems to be the plant growth regulators (Halperin, 1969; Greshoff, 1978). There are two classes of PGR most commonly used, namely, CK's and auxins. Generally, the CK's have been classified as regulators of cell division and the auxins as regulators of cell elongation (Roberts and Hooley, 1988).

Although, a small fraction of CK's may be synthesized by shoot grown *in vitro* (Koda and Okazawa, 1980), roots are the principal site of CK biosynthesis (Hu and Wang, 1983). Three CK's frequently used in tissue culture studies i.e. K, BAP and IPA (N^6-(2-isopentenyl)-adenine

or 2ip). Among these BAP is most effective for meristem, shoot tip and bud cultures, followed by K. IPA has been used less frequently (Nair *et al.*, 1979). The effectiveness of a particular PGR is reflected by the frequency of their usage. Hu and Wang (1983) and Thomas and Blakesley (1986), found that BAP usage in micropropagation accounted for almost 65 % of species propagated commercially . While the employment of K and IPA were 22 - 23 % and 4 -9 % respectively. BAP is the most popular in the commercial sector because it is cheap and requires no filter sterilisation and can usually be used in relatively low concentrations, less than 5 micromolar (Thomas and Blakesley, 1986). The low percentage usage of IPA and zeatin is mainly due to its high cost and limited availability (Hu and Wang, 1983). It is recognised that the requirement for plant growth regulators varies considerably and it is dependent on the species and the level of endogenous growth substances inherently present in the culture cells (Murashige and Skoog, 1962; Schenk and Hildebrandt, 1971). For example for the Ericaceaous species IPA is the most effective (Hu and Wang, 1983). In some species the addition of CK is not necessary for their establishment in culture, presumably enough endogenous CK is present in the explants. Moreover in some species like *Rubus, Solanum* and *Fragaria* adventitious roots readily regenerates in culture, these provide the necessary CK when the residual CK present in shoots is depleted. However, these species (Hu and Wang, 1983) did not produced multiple shoots in culture. At the shoot proliferation stage CK is needed to suppress the apical dominance of shoots, thereby enhancing the branching of lateral buds from leaf axils. Again the best concentration of CK to develop multiple shoots varies according to the species. In general, BAP is the most potent CK for stimulating axillary

shoot proliferation, followed by K and IPA (Bhojwani, 1980a; Kitto and Young, 1981; Lundergan and Janick, 1980).

For normal shoot growth, the presence of auxin is necessary. When relatively large shoot tip explants from actively growing plants are used, exogenous auxin is not always needed in the establishment stage. But the presence of auxin is sometimes reported (Dale, 1975) as not essential but is beneficial for the growth of the culture. There are several plant growth regulators possessing auxin-like properties commonly used in plant tissue culture. Indole acetic acid (IAA), IBA, NAA and 2,4-dichlorophenoxyacetic acid (2,4-D) are the most frequently used in plant tissue culture. According to Hu and Wang (1983), IAA is the weakest of the four and easily rendered inactive by light or by tissues with high IAA-oxidase activity. IBA is more effective than IAA and 2,4-D is the most potent of the four. 2,4-D stimulates callus formation and is strongly antagonistic to organogenesis. Of the four, NAA is routinely used by most laboratories for meristem shoot tip and bud cultures. GA was only used in 17 % of the species (Hu and Wang, 1983). This suggests that this hormone is sufficiently present in many explants.

The presence of auxin during the shoot proliferation stage is thought to neutralize the suppressive effect of high CK concentrations on shoot elongation thereby restoring normal shoot growth (Lundergan and Janick, 1980).

At the rooting stage, that is the stage III, the hormonal requirement is the opposite of that required for shoot proliferation (Stage II) i.e. for root initiation there should be low CK and high

auxin combinations in the medium. Hussey (1986), stated that CK's inhibited rooting, with BAP being particularly strong in this regard.

(d) Agar and acidity.

The use of agar as the carrier material to solidify the medium for supporting the explant during organogenesis in plant tissue culture is standard. The choice of a suitable gelling agent in the appropriate concentration may help to control vitrification in broadleaved tree species (Pasqualetto *et al*, 1986). Raising the agar concentration, however, very often lowers propagation rates.

Generally, the concentration of agar used is between 0.4 to 0.8 % at all stages of micropropagation *in vitro*. However, Lane (1979) reported in certain sensitive species such as *Prunus* that 0.6 % agar may result in poor root growth. Also Kitto and Young (1981) observed that there is an inverse relationship between rooting ability and the concentration of agar. One reason for poor rooting in an agar medium may be due to poor aeration and slow rates of diffusion of toxic metabolic wastes released by growing tissue (Hu and Wang, 1983). Two methods were suggested to overcome the inhibitory effect of agar medium: (i) by addition of fine powdered activated charcoal (AC) (ii) use of a liquid and filter-paper-bridge system in place of agar.

The pH of the medium is normally adjusted to between 5 - 6 before sterilisation. The value of pH between 5.7 and 5.8 is recommended by Murashige and Skoog (1962), as this pH range was suitable for the maintenance of all the salts in a soluble form.

(ii) Incubation conditions.

The two most important incubation conditions for *in vitro* cultivation of plants are temperature and light (Murashige and Skoog, 1962). Studies to reveal the optimal incubation conditions of these factors are very few but a number of authors report that they can be important in some species (Hussey, 1986). Optimum conditions, as expected, will vary with species and the usual environment where the plant originated offers some guidelines as to the selection of temperature (Hu and Wang, 1983)

The majority of workers have adopted the same temperature conditions throughout the whole process of micropropagation which generally varies between 20 - 28°C (Hu and Wang), with the majority at 25°C (Hussey, 1983). Cultured isolated buds of *P. abies* (Norway spruce) (Von Arnold and Erikson, 1979) formed adventitious shoots at temperatures between 15 - 30°C, but the optimum temperature was 20°C. Tropical species require higher temperatures, the optimum for date-palm being 27°C (Tisserat, 1981).

Lane (1979b) reported that when the temperature is above 28°C, condensation of water may occur on the plants and the container wall, which may restrict growth.

Plant tissue cultures are normally grown under fluorescent tubes at intensities 1000 - 10,000 lux for 16 hours daily (Hu and Wang, 1983). However, Hussey (1986) stated that intensities vary between 1000 - 5,000 lux. According to Murashige (1974), higher light intensities up to 1 klx have been found to be beneficial, especially at the rooting stage. As for the temperature requirement, the exposure and light intensities will depend on the species under culture.

2.3.2.3 General problems encountered in tree tissue culture

In vitro propagation systems for tree species will, without doubt, become the method of the future for cloning desirable genotypes. Not least of which, because it is an extremely space saving propagation technique (Boxus *et al*, 1977; Wang and Hu, 1982). However, there are problems associated with forest tree tissue culture that need to be considered. Firstly, contamination from microorganisms, secondly, the inhibitory effects of polyphenolic and other compounds exuded into the culture medium by the explants and finally, the instability of some of the regenerated plants (epigenetic and somaclonal variations). The third problem is not relevant to plants derived from meristems, shoot tips or bud cultures.

2.3.2.3.1 Contamination

The problem of contamination in tree tissue culture is a major one, and begins at the plant collection stage and continues until the end of the micropropagation process, i.e weaning and hardening of the regenerated plants. Contamination is more severe with tropical species (Smits and Struyken, 1983) with contamination levels as high as 95 % when cultures from a mature explant source were being established. The infection was caused by a fungus that lives in the vessels of the *Dipterocarpus grandiflorus* (keruing) leaf. Complete disinfection of many woody species can be extremely difficult particularly if the explant is brought in from the field. Other examples of micro-organisms that invade the tissues of the plant are reported in dormant winter buds of pecan trees (Knox and Smith, 1980) and in apple (Andrews, 1980). In this case, decontamination is only obtained by culture of the isolated apical

meristem as employed for virus elimination (Hussey, 1986).

The microorganisms that survive the surface sterilisation process, belong to a particular species of fungi and bacteria. They appear usually within one or two weeks after incubation, and outgrow the explants in a culture medium containing a high sucrose level, they eventually kill the explant. Sometimes, slow-growing bacteria such as *Pseudomonas spp.*, *Erwinia spp.* and *Bacillus subtilis* will remain in a small number close to the explants and quite invisible . It may not cause any loss in terms of shoot production, but their presence may affect the vigour of the proliferating shoots , cause chlorosis on the plantlets and inhibit rooting (Knauss and Miller, 1978; Hussey, 1983 and 1986).

Various solutions have been suggested to overcome this problem mainly involving treatment with antibiotics and fungicides on the source explants, or the inclusion of antibiotic compounds in the culture medium. Two antibiotics, tetracycline and rifampicin were found to be most effective in eradicating bacteria (Young et. al., 1984). A mixture of an antibiotic and a systemic fungicide such as 0.1 % streptomycin + 0.1 % benlate is another alternative. The use of various different sterilsation procedures have also been recommended, such as a two-step sterilisation process, or the use of surface sterilants such as mercuric chloride, benzalkonium chloride or hydrogen peroxide. However, it would appear that contamination is still one of the most severe problems affecting tree tissue culture programmes.

2.3.2.3.2 Polyphenolic compounds

The phenomenon of explant browning affecting the culture initiation of woody explants is frequently seen in the culture of fruit trees (Jones, 1983) , tropical hardwoods (Smits and Struycken, 1983), date-palm (Tisserat, 1979), and teak (Gupta et al., 1982). This browning subsequently leads to explant death. Polyphenolic compounds are produced when plant tissue is damaged either at the dissection process or during surface sterilisation these compounds are oxidised and the tissue turns brown or black. The oxidation products also darken the media. Various alternative solutions to overcome this serious problem have been proposed, usually by supplementing in the medium with antioxidant such as cysteine, ascorbic acid, dithiothreitol (DTT), polyvinyl pyrrolidone (PVP) and activated charcoal (Jones, 1983). Alternatively, explants have been presoaked in antioxidants before planting into the culture medium, or have been initially incubated in reduced light conditions or in total darkness, or explants are frequently changed into fresh medium when browning is encountered (Hu and Wang, 1983).

3. OBJECTIVES

The main objective of the first part of my study was to investigate the factors that influence after-ripening and dormancy breaking of *S. aucuparia* L. (rowan). Some of the information regarding dormancy breaking had been established by earlier workers (see part 2.2.1.5) . However, there are many unanswered questions regarding dormancy breaking, in particular, the nature of embryo dormancy that the seed possesses. Therefore, initially studies (Section III.1) aimed to explore various dormancy breaking treatments to compare and confirm earlier findings. Methods of germinations reported (ISTA, 1985) for this species need to be reviewed in the light of recent suggestions (Gordon and Rowe, 1982) and findings (Lenartowicz, 1988) on the best germination procedures.

Exogenous applications of PGRs both *in vivo* and *in vitro* have not been attempted before on *Sorbus aucuparia* seed or embryos; other studies involving PGRs in other species have improved our knowledge on the nature of their dormancy. The regulatory role of endogenous PGRs in the control of seed dormancy and germination is now well established, although the mechanism by which this control is brought about is still not fully understood. Changes in the levels of endogenous PGR's, in particular, the CK's have not been studied during after-ripening by cold stratification in this species. This study attempts to explore and gather further information on the effects of PGR and growth media on the dormancy of *Sorbus aucuparia*. This species offers excellent material for a study of the factors involving dormancy as both seedcoat and embryo dormancy are present at the same time. Moreover, the seedcoats could be easily excised and the mature embryo could be isolated for

testing.

In the second part of my study (Section III.2), the primary intention was the development of a suitable micropropagation system in *S. aucuparia* using seed materials as the initial explant source. The aim of the experimental work was to ascertain the optimum PGR's concentrations for shoot proliferation and root induction *in vitro* and to ascertain whether direct rooting was possible.

SECTION II.

GENERAL MATERIALS AND METHODS

1. GENERAL FLORAL BOTANY AND DEVELOPMENTAL STUDIES

Initial studies on UK broadleaved trees investigated flowering, fruiting and seed losses of three species *Acer platanoides L, A. pseudoplatanus L.* and *Sorbus aucuparia L.* Four sites met the study requirements. These were the Dalmeny Estate at South Queensferry, Buccleuch Estate at Dalkeith, Hermitage of Braid (located between Morningside and Liberton), Edinburgh and the Calderwood-Almondale Country Park near East Calder. All the four sites were within a 15 mile radius of Edinburgh City.

Reconnaissance of the areas were carried out extensively with the aim of finding suitable trees of at least 20 years old but avoiding tall trees for practical reasons. Trees were selected that grew in the open canopy within the forests or along forest roads and paths.

1.1 Seed Production of *Acer platanoides* L. and *A. pseudoplatanus* L.

On 3 September 1986, from a single tree growing at the edges of an open field in the vicinity of Dalmeny Castle, a total of 194 racemes of Norway Maple *(A. platanoides L.)* fruits were sampled. On the 8th September 1986 a total of 137 racemes of Sycamore *(Acer pseudoplatanus L.)* were sampled from a single tree also located in the area of the park. Each raceme was individually plucked either by hand or by a pruning cutter. Fruits were kept intact on their raceme and were scored in the laboratory for the following criteria:

(a) Total number of fruits collected

(b) Total number of fruits with filled seed

(c) Total number of fruits with empty seed

(d) Total number of diseased seed

From the above values estimates of the entire fruit and seed production were derived for each particular tree.

1.2 Seed Production of Rowan (*Sorbus aucuparia* L.)

This study was carried out at the Hermitage of Braid. A mixed woodland of semi-opened canopy comprising of tall specimens of sycamore (*A. pseudoplatanus*), elms (*Ulmus glabra)*, oak (*Quercus robur*) which occupied the top canopy. Two **Rowan** trees A and B, were chosen for this study with the aim of investigating fruit production and seed set on individual inflorescences (corymbs). The trees were within 10 - 30 metres of each other, 4 - 5 metres tall, with a diameter breast height (DBH) between 10 -15 cm and were approximately 12 - 15 years old. For tree A, 9 inflorescences or corymbs were tagged and 8 for tree B. Their periodic recordings for flower opening, flower drop and seed set were monitored.

1.2.1 Scoring for flower number

Flower position within a corymb was assigned a number one being the lowest, two the next lowest and so on (Fig. II.1). In this way, for each plant sampled the position of each flower and hence the resultant fruit set was quickly and accurately recorded for every flowering corymb. Flowering began in early May. The positions of the flower buds were mapped by assigning numbers (see Fig.II.1) so that its development and history could be periodically monitored by succesive observations.

Data collected for each corymb included the initial number of buds prior to flower opening, numbers of aborted buds, flowers and fruit

set. Bud or flower losses due to predation primarily by a larval stage of a species of chalcid fly were also counted, based upon its characteristic feeding marks on the buds or flowers. Anthesis was considered to have occurred when the stigmas and style changed colour from cream to brown. After anthesis observations and countings were done once a month until the fruit was matured in September.Detection for the presence of pollen tube in ovaries of abscised flowers were performed following methods by Martin (1959) and later modified by Smith (1982).

2. STUDIES ON SOME ASPECTS OF SEED DORMANCY IN *S.AUCUPARIA*

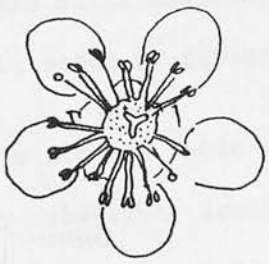
2.1. Seed source

Rowan (*Sorbus aucuparia L.)* seeds from 1985 and 1986 harvests were obtained from British Seed Houses Ltd. Rowan fruits were also collected from a mature tree growing within the campus of the University of Edinburgh or from marked trees growing at the Hermitage of Braid. The collections were made during 1986, 1987 and 1988 fruiting seasons either by hand or a long arm pruner.

2.2 Extracting, cleaning and storing

Rowan berries collected were immediately processed for their seeds. The berries were placed in a sieve and squashed with a presser board to break down any flesh. The residual mass was then dropped into a beaker or bowl of warm water leaving it to soak for a few hours. The fleshy material was decanted off, leaving seeds at the bottom. This process was repeated many times until all seeds were thoroughly cleaned.

Seeds were left to dry for two or three days on a plastic tray at room temperature (approximately 20°C). When dried, usually, there was



Single rowan flower (4x)

Fig. II.1 : A sketch of a corymb of *Sorbus aucuparia* L. A characteristic feature of the genus *Sorbus* is the grouping of the flowers and their resultant fruits in clusters that are made up of one main stalk and many smaller ones. In the diagram above each smaller stalk and their branches are numbered. The numbers within each circle are an example of the total number of flower buds in one of the corymbs.

some skin still adhering to the seed. These dried skins were separated from the seeds by squeezing.

The cleaned seeds and the seeds that were purchased from the Seed Company (British Seedhouses Ltd.) were kept in separated plastic bottles (screw-capped), labelled and stored at 4°C. Seeds used for various studies were taken out when needed from the bottles. Good-sized seeds without evident damage to the testa were selected by screening it through a table-top magnifying-glass.

2.3. Sterilisation

Unless otherwise stated, all seed used for the experiments were surfaced sterilised with 1% (v/v) sodium hypochlorite solution for 10 - 15 minutes , to which a few drops of Tween 20 had been added as a wetting agent. This was followed by three washings with sterile distilled water.Seeds were then soaked in sterile water for two days changing the water twice daily. After imbibition the seeds were again resterilised with 1 % hypochlorite.

For embryo surface-sterilisation the immersion time in hypochlorite solution was reduced to 10 seconds followed by three washings in sterile distilled water. Excess water was removed by placing the seed in between two sheets of sterile filter paper.

All instruments used were wrapped with aluminium foil and sterilised by autoclaving at 120°C and 1.1 Kg/cm²C for 15 minutes.

Most work was carried out in a laminar flow cabinet (Flow Labs. Irvine, Scotland) which was prepared by switching on 30 minutes before use and ensuring that at least the sides and bench surfaces were

wiped with 'Dettol' solution or the interior of the cabinet sprayed with 70% alcohol and allowed to dry. As a table-top magnifying glass was used to aid excision this was also sprayed with alcohol (70 %) and wiped dry. When necessary surgical gloves were worn otherwise it was sufficient to spray hands and arms with 70% alcohol or methylated spirit.

2.4 Seed extraction and *in-vitro* cultivation

Using scalpel and forceps the seeds were prised out. Seeds were surfaced sterilised as descibed above. The two seed coats were removed aseptically. Embryos or whole seeds were placed into 60 ml specimen jars containing 20 ml half-strength MS medium with 1 % agar. Each jar contained five seeds or embryos and each cultivation was replicated five times. Jars were transferred to a Gallenkamp growth cabinet at 20°C and continuous white fluorescent light as in germination test (GT). Observations were performed daily after 7 days of incubation.

2.5 Tests of viability

The aim was to make a quick estimate of the viability of rowan seeds received from the seedmen and for this purpose, two procedures were employed namely, the **biochemical tetrazolium test** and the **embryo-excision test.** All tests followed ISTA rules for seed testing (1985).

2.5.1 Tetrazolium Test Procedures

(i) Reagents

An aqueous solution of 1% 2,3,5-triphenyl tetrazolium chloride of pH 6.5 - 7.5 was used and was buffered to obtain the correct pH range. The buffer solution was made up as follows.

Two solutions were prepared:

Solution 1: 9.078g KH_2PO_4 was dissolved in 1000 ml of water

Solution 2: 9.472g Na_2HPO_4 was dissolved in 1000 ml of water

The solutions were mixed, two parts of solution 1 with three parts of solution 2.

(ii) Preparation of the seeds before staining

About 300 - 400 seeds were soaked in distilled water at room temperature for 18 hours. Pre-moistening was necessary prior to staining as imbibed seeds were less fragile and more easily cut and punctured. It was neccessary to cut in order to expose the seed tissues prior to staining thus allowing easier penetration of the tetrazolium solution. Procedures for exposing the internal tissues have been standardised (ISTA,1985).

Seed coats were removed using a scalpel, needles and forceps. For *Sorbus aucuparia L.* seeds, a cut was made transversely 1/3 from its distal end (at the hilum) opposite the radicle.

Once prepared the seeds were kept moist until one complete replicate had been prepared. The replicates were then immersed in tetrazolium solution.

(iii) Staining time

Each replicate of the bi-sected seeds in tetrazolium chloride solution,was kept in the dark by covering the beaker with aluminium foil, and kept at room temperature for 24 hours.

(iv) Evaluation

The evaluation was performed according to an ISTA (1966) evaluation

scheme as follows:

Sorbus aucuparia L. (rowan)

The following seeds were considered viable:

(a) completely stained embryo.

(b) embryo showing an unstained point at the radicle tip.

(c) embryo showing unstained spots, on the cotyledon opposite the radicle, allowing superficial necrosis affecting up to 1/2 of the cotyledon and pervading necrosis affecting up to 1/3 of the cotyledon.

(d) cases (b) and (c) combined

2.4.2 Excised-Embryo Test

This test were performed on *Sorbus aucuparia L.* seeds to determine the approximate viability and to compare with the 'biochemical test' of tetrazolium staining.

2.4.2.1 Procedure

(a) 200 seeds were surface sterilised as described earlier (see section II.2.3). They were soaked in sterile distilled water for 48 hours, the water was changed twice daily. Using sterile needles, scalpel and forceps the seed coats were removed surgically from the embryo. Throughout the operation the embryos were kept moist in the laminar flow cabinet.

(b) The embryos were then placed onto moistened filter paper Whatman No.1 in plastic petri dishes in 12 replicates of 10 embryos each. The petri dishes were sealed with parafilm.

(c) The excised embryos were then incubated at a constant temperature of 20°C for up to 14 days with 8 hours of light daily. Petri dishes were

examined daily, decayed embryos were discarded as soon as they were found.

2.4.2.2 Evaluation

The following categories were considered viable,

(a) Developing embryos

(b) Embryos with one or more cotyledons exhibiting growth or greening.

(c) Embryos remaining firm, slightly enlarged and either white, green or yellow.

The following were considered non-viable,

(a) Embryos which rapidly developed severe mould.

(b) Embryos which discoloured becoming black or brown or watery in appearance.

2.4.3 Germination Test (GT)

The determination of the germination behaviour of a batch of seeds required the performance of germination tests in which samples of seeds were allowed to germinate in a petri dish or a container under controlled conditions.

The tests were performed under sterile conditions. Filter papers and all instruments used for the test were sterilised as described in section II.2.3. The tested seeds or embryos were placed aseptically on either a 9 cm or 3.5 cm petri dish, which contained two layers Whatman No.1 filter papers and 5 ml or 1 ml sterile distilled water respectively. The dishes were sealed with parafilm and placed randomly into a Gallenkamp growth cabinet. The growth cabinet was set up at 20°C and continuous white fluorescent light at a photon flux density of 55 umol $m^{-1}s^{-1}$ for one month (GT). Observations were made weekly on cotyledon

greening, root and shoot growth. Protrusion of the radicle through the testa and the earliest sign of geotrophic curvature were considered to be evidence of germination.

3. MICROPROPAGATION OF ROWAN (*S. AUCUPARIA* L.)

3.1 Explant materials

3.1.1 Seed embryos

Fresh seeds were obtained from trees situated at Kings Building, University of Edinburgh. Selected seeds were surface sterilized (section II.2.3) and embryos were prepared as previously described (section II.4.2). The embryos were placed on the basal medium (section II.2.4.4) supplemented with 9 concentrations of cytokinins (BAP) and 10 concentrations of auxins (IBA) in each and every combination (section III.2.1, Table III.2).

3.1.2 Seedling explants

Two ages of seedling explants were used to investigate the influence of CK and auxin on shoot proliferation, both were derived from seed explants ; (i) two-months old and (ii) 8-months old. The latter were those obtained from rowan embryos treated with various concentrations of BAP (**Experiment 1**, Section III.2.1), whereas the former were raised from **Experiment 2a** in the same section as above.

3.2 Shoot excision

Excised shoots employed in all the experiments were specially prepared in a laminar flow cabinet. Each shoot was cut at an angle to expose as much as possible of the base of the stem to provide a better intake of PGR's and nutrients from the medium where they were grown. Although PGRs may be absorbed through the bark, most of the hormone will be taken up

3.3 Media preparation The preparation of media was performed as follows :

(a) Preparation of stock solution for growth regulators

The plant growth regulators used for these studies were; (i) cytokinins namely 6-benzylaminopurine (BAP) and Kinetin (K) (ii) auxins namely Indole-3-butyric acid (IBA), 1-napthaleneacetic acid (NAA), Indoleacetic acid (IAA) (all obtained from Sigma Ltd.). Stock solutions were prepared by dissolving 25 mg of growth regulator in a small quantity 1M NaOH, for BAP, IBA, or 1M KOH, for K or 100 % ethanol, for NAA, which was subsequently made up to 25 ml with distilled water to give a 1mg/ml concentration stock solution.

(b) Preparation of the nutrient medium and other additives.

A premixed powder of Murashige and Skoog (MS) culture medium (Flow Laboratory, Ayr) was weighed to half recommended strength (2.354 mg/l) to which was added sucrose at either 0, 0.8 or 3 % (depending on the experiment), Difco agar(0.6 %) and 750 ml of distilled water in a one litre flask. The suspension was heated to near boiling to dissolve the agar on a magnetic stirrer hot-plate. The pH was measured and adjusted using 1M NaOH or 1M HCl to 5.8 for all experiments. The solution was then made up to 1 litre by adding distilled water. The media was divided into smaller volumes either 100 or 250 ml depending on the number of replications and the volume per replicate required.

(c) Appropriate quantities of cytokinin and/or auxin and a drop of Tween 20 was quickly added to the flasks containing the solution previously described by using a micropipette. The flasks were well shaken to facilitate complete mixing of the nutrient mixtures and PGR's.

(d) The mouth of the flasks were enclosed with two layers of aluminium foil, and were sterilised by autoclaving at 120°C and 1.1 kg/cm² for 15 minutes.

(e) The medium was allowed to cool to around 60°C and subsequently maintained at 60°C in a water bath to prevent solidification before use. The medium was dispensed into culture vessels aseptically in a laminar flow cabinet previously sterilized as described in section(II.2.3), and allowed to cool at room temperature, culture vessels were stored at 4°C before use.

(f) Sterile techniques were performed according to the methods described in section II.2.4.2.I (a).

3.5 Environmental conditions

The light regime (photoperiod and intensity), was kept constant for all stages of *in-vitro* plant regeneration. The cultures were incubated at an average temperature of 20±1°C and a photoperiod of 16 h in Gallenkamp or Conviron EF-7H growth cabinets.

3.6 Statistical analysis

All experiments unless otherwise stated were arranged according to a completely randomised design on shelves of the growth cabinets or greenhouse benches. The data were analysed employing the GENSTAT IV analysis of variance computer program, located on a VAX PDP11 at The Edinburgh School of Agriculture except for section IV.3.4.2 in which case data were analysed using Statistical Analysis System (SAS) on an IBM-compatible, Hewlett-Packard, Vectra Computer System-Model 20 and Series No. D1180A.

SECTION III.

SPECIFIC MATERIALS AND METHODS

1. EXPERIMENTAL STUDIES FOR BREAKING OF SEED DORMANCY IN *S. AUCUPARIA*

1.1 The effect of stratification at different temperatures

Rowan seeds used for these studies were either surface-sterilised or left unsterilised. Treatment comprised incubation at 1°C, 4°C, 10°C, 15°C and 20°C. The petri dishes were then placed randomly into incubators at the various temperatures in the dark. Germination recordings were made weekly, infected seeds were immediately removed and the petri dishes were watered when necessary.

Experiment 1: To investigate after-ripening in various stratification temperatures.

Thirty, sterilised rowan seeds, were placed aseptically on a 9 cm petri dish. Five such petri dishes were wrapped in a polythene bag and sealed. Each temperature treatment consisted of 40 such petri dishes and were left to stratify for up to 36 weeks.

Experiment 2: To investigate after-ripening in various stratification temperatures without first surface sterilising the seeds.

One hundred unsterilised rowan seeds from British Seed Houses Ltd. or from Hermitage of Braid were placed using a vacuum planter onto a petri dish layered with filter paper. Sterile water (5ml) was added. Each treatment temperature consisted of five replicates, each sealed with parafilm to reduce water loss and left to stratify for up to 32 weeks.

Experiment 3: To investigate after-ripening at 4°C.

Rowan seeds collected from a single tree at Kings Building were surface

sterilised. Fifteen seeds were placed aseptically onto a 9 cm petri dish previously layered with filter paper. Five ml of sterile water was then added. There were 25 replicates and all were left to stratify at 4°C for 22 weeks.

1.2 The effect of the duration of warm temperatures on seed germination preceding a cold temperature treatment.

Experiment 4 : To investigate whether after-ripening occurred at 10°C, 15°C and 20°C at different periods of stratification.

This experiment was set up simultaneously and similar, to 1 and 3 above. However, at monthly intervals, a sample, of 5 x 30 seeds was removed and tested for germination as described in section II.2.4.3.

Experiment 5: The effect of six months warm either at 10°C 15°C or 20°C preceding a cold stratification at 4°C.

Seeds incubated at 10, 15 or 20°C **(Experiment 4)** for more than six months were subject to incubation at 4°C.

Experiment 6: To investigate the effect of two weeks alternating temperature on the after-ripening of rowan.

Rowan seeds collected from Kings Building were sterilised. Five seeds were placed aseptically on a 4 cm petri dish layered with 2 Whatman No 1 filter papers. Sterile distilled water (1 ml) was added. Twelve replicates were taken, each was sealed with parafilm. All dishes were placed at 20°C in the dark for two weeks, they were then placed at 4°C for 8 months.

1.3 The effects of various combinations of alternating temperature of warm and cold preceding stratification at 4°C.

Experiment 7 : The effect of various combinations of warm and cold preceding stratification at 4°C.

Non-germinated seeds from **Experiment 1** were restratified with various combinations of times and temperatures as summarised below (Table III.1).

1.4 Analysis of Data

Data from the above experiments were subject to analysis of variance. For all experiments the following measurements were taken:

(a) Total Germination Percentage(TG) - the percentage of seed germinated until the termination of stratification.

(b) Median Germination Week (MGW) - the number of weeks of cold treatment that it took to reach 50 % of TG.

(c) Beginning of the "Intensive Germination Period" (IGP) - the number of weeks elapsed from the start of the cold treatment until 10 % TG,had been attained.

(d) Duration of IGP - The difference between the number of weeks of cold treatment that it took to achieve 10 % and the time taken to achieve 90 % of TG.

1.5 The effect of stratification on different media.

Experiment 8 : Investigation of the influence of four different media on after-ripening at 4°C stratification temperature.

Rowan seeds used for this study were obtained from British Seed Houses Ltd. and all work performed under aseptic conditions in a laminar flow cabinet.

Table III.1: Summary of treatments to break the dormancy of *S. aucuparia L.* seed, using combinations of time and temperature , with or without light. All treatments ended with restratification at 4°C until germination occurred.

Seed Batches	Treatments
A. 1°C	(a) 1m @ 1°C, 3m @ GT and 7m @ 4°C
	(b) 2m @ 1°C, 2m @ GT and 7m @ 4°C
B. 4°C	(a) 1m @ 4°C, 1m @ GT, 1m @ 4°C, 1m @ GT and 8m @ 4°C
	(b) 2m @ 4°C, 1m @ GT, 2m @ 10°C and 8m @ 1°C
C. 10°C	(a) 1m @ 10°C, 1m @ GT, 2w @ 4°C, 1m @ GT and 9.5m @ 4°C
	(b) 3m @ 10°C, 1m @ GT, 2w @ 4°C, 1m @ GT and 9.5m @ 4°C
	(c) 4m @ 10°C, 1m @ GT and 9.5m @ 4°C
	(d) 5m @ 10°C, 1m @ GT and 9.5m @ 4°C
D. 15°C	(a) 1m @ 15°C, 1m @ GT, 1m @ 15°C,1m @ GT and 8.75m @ 4°C
	(b) 2m @ 15°C, 2w @ 4°C, 1m @ GT and 8.75m @ 4°C
	(c) 3m @ 15°C, 1m @ GT and 8.75m @ 4°C
	(d) 4m @ 15°C, 2.5m @ GT and 8.75m @ 4°C
E. 20°C	(a) 2m @ 20°C , 1m @ GT, 2w @ 4°C, 1m @ GT and 10m @ 4°C
	(b) 4m @ 20°C , 2.5m @ GT and 10m @ 4°C

m = month/months w = week/weeks

GT = 20°C temperature condition with continuous light.

Three metal boxes (20 x 20 x 10 cm) were lined with aluminium foil and each were filled with a different substrates, namely; sand, vermiculite or peat, to a depth of 5 cm. The boxes were placed in the

oven for three hours at 150°C and the contents turned regularly for the first hour to ensure even drying and to prevent overheating. Two polythene bags (20 x 10 cm) were also used to contain the seeds during stratification. These polythene bags were sterilised by turning inside out and sprayed with 70 % alcohol and later left to dry in a laminar flow cabinet in U.V. light for 30 minutes.

Sterilised distilled water (2 litres) were poured into the boxes of substrates and left to soak until the substrates appeared homogeneously moist. Excess water were drained out by tilting the boxes. The seeds were then mixed well into each substrate. Alternatively seeds were placed in the polythene bags and enough water added (5 ml) to keep the seeds just moist, the bag was then sealed. Seeds were kept at 4°C in an incubator (Swan,cooled incubator, model 13-80,Type CB/MK II).

Germinability of all lots was periodically determined after 0, 4, 6, 8, 10 and 12 weeks stratification. The test procedures followed ISTA rules except the number of seeds and replications were modified. After stratification germinated seeds from each media were carefully sorted into even sized seedlings, large or small seedlings were discarded and grown on petri dishes in an incubator at 20°C with constant light.

Each seed test consisted of 10, 4 cm petri dishes each with either five seeds or five embryos that were placed on top of filter paper, Whatman No.1, moistened with 1 ml of sterile distilled water. The petri dishes were sealed with parafilm. Hypocotyl length, root length, shoot length and the number of leaves produced after seven and twenty-eight days incubation were recorded. All seeds and embryos were incubated at 20°C with continuous light.

After the 12th and 14th week of stratification a random sample of each substrate was taken out and the first 200 seeds were counted for percentage germination. Besides the above investigation ,a study of the behaviour of dormant embryos stratified at 4°C , at various period in polythene bags was carried out concurrently.

Experiment 9: To investigate the behaviour of dormant embryos and germinated seedlings of seeds previously stratified at 4°C.

The periodic tests for germinability of excised embryos from seeds that had been stratified for different periods at 4°C in all media, effected various growth responses to the embryos. These responses were recorded after , 1 week and 4 week incubation, for each different stratification period. Six parameters were measured:

A. No change to both cotyledons (white/cream in colour)

B. Semi-greening of a cotyledon and one cotyledon remaining unchanged (cream/white).

C. Semi-greening of both cotyledons.

D. Full greening of a cotyledon and one cotyledon remaining unchanged (white/cream).

E. Full greening of both cotyledons

F. Full germination of embryos, producing radicles, hypocotyls and shoots.

The above parameters, both in percentages and transformed values were subjected to the analysis of variance.

There were two types of data generated from the above experiments, namely, the behaviour of dormant embryos and the behaviour of the germinated seedlings.

1.6 Cultivation *in-vitro* and the effect of Plant Growth Regulators (PGRs)

Experiment 10: To investigate the effect of applied cytokinins and auxins on dormant seeds or embryos.

Seeds from British Seed Houses Ltd. were surface sterilised as previously described (Section II.2.3) and were imbibed for 48 hours in sterile distilled water which was changed daily. The two seed coats were removed aseptically. Embryos or whole seeds were placed into 60 ml specimen jars containing 20 ml half-strength MS medium with 0.8 % agar and various concentrations of either GA_3, GA_4, GA_7, IAA, BAP, NAA or IBA as follows: 0, 0.1, 1, 2, 3, 4, 5 and 10 mg/l. Each jar contained five seeds or embryos and each treatment was replicated five times. Jars were transferred to a Gallenkamp growth cabinet at 20°C and continuous white fluorescent light of similar photon flux density as in the earlier experiment. The incubation period was one month and the observation were performed weekly on cotyledon greening, root and shoot lengths.

Experiment 11: To compare the development of embryo growth on paper substrate wetted with BAP at various concentrations.

Excised embryos were germinated aseptically in small petri dishes (4 cm diameter) containing 0.7 ml of the following BAP solutions: 0, 0.1, 1, 2, 3, 4, and 5 mg/l. Five embryos were planted on top of two layers of Whatman No 1 filter paper. The experiment was replicated five times and were incubated and recorded as in **Experiment 10.**

1.7 Infusion of plant growth regulator into seeds.

Experiment 12: To investigate whether BAP could be infused into dormant rowan seeds .

Organic infusion or permeation was performed according to the method of Khan, Braun, Tao, Muller and Bensin (1976). Various concentrations of BAP were prepared by appropriate dilution of a 1 mg/ml BAP stock solution. Dry rowan seeds were treated for periods of 1 hour or 2 hours by immersion in sealed glass jars containing the solution of BAP either at of 0, 1, 5, 10, 50, 100 and 200 mg/l at room temperature (20°C). A shaker was used during the infusion process to keep the solution and the seeds agitated so as to accelerate the process. A germination test was performed in 4cm petri dishes layered with filter papers moistened with both hormone solution of the same treatment concentration as well as with sterile distilled water as a control. The germination tests consisted of 5 seeds per dish and replicated 10 times for each hormonal concentration tested.

Experiment 13: To investigate whether BAP solution would penetrate the mesocarp into the embryos.

Selected rowan seeds obtained from British Seed Houses Ltd. were soaked for 48 hours so make testa excision easier and making it possible to leave the mesocarp intact. Five seeds with the mesocarp intact and five excised embryos were germinated separately in 4 cm petri dishes with 2 mg/l BAP added. There were five replicates of each treatment. All work was done in sterile conditions. The conditions of the germination test was as described in Section II.2.4.3.

1.8 Mechanical restriction of embryo growth by seed coverings

Experiment 1: To investigate the role of the seed coats of *S. aucuparia* seeds in the dormancy process.

Fresh seeds collected from the K.B. campus (see section II.2.1) were surface sterilised in the usual manner and later soaked for 2 days with a daily change of sterile water.

Four preparations of seed were set up aseptically in a laminar flow cabinet.

(1) Whole seeds

(2) Embryos with mesocarp intact and

(3) Embryos with mesocarp nicked on one side with a scalpel about 2 mm long avoiding any injury to the embryos.

Germination tests (as in Section II.2.4.3) were then performed on 4 cm petri dish on top of filter papers with 1 ml of 2 mg/l BAP added. Five seed per dish, replicated 10 times. Observations were made on root and hypocotyl length, shoot height and number of leaf formed.

1.9 Hormonal changes in seed during after-ripening by prechilling

Experiment 1: To detect the presence of cytokinin at different periods of cold stratification.

Seed of rowan obtained from British Seed Houses Ltd. were stratified at 4° C in polythene bags, following soaking for 24 hours and surface-sterilisation.Samples were taken at intervals of 4, 6, 8 and 10 weeks of stratification and cytokinins were extracted from seed testas, membranes and embryos, as described in Horgan (1978).

1.9.1 Extraction of cytokinin

Testas, membranes (mesocarps) and embryos were separated. Each seed part was weighed and frozen in liquid nitrogen and immediately dropped into methanol:chloroform:formic acid (12:5:3, v/v) at -20°C (10 ml of solvent per gram fresh weight). After 24 hours at -20°C, to inactivate phosphatase, the tissue and solvent were homogenised at 20°C and centrifuged at 10,000 g (max.) for 30 min. Chloroform and water were added until the ratio of methanol:chloroform:formic acid was 12:11:10 (v/v), this helped to clear away lipid. The phases were separated by centrifugation (10,000 g for 30 min) and the aqueous phase was evaporated under reduced pressure at 25 - 30°C. The residue was then dissolved in ethanol:water (1:1, v/v) for thin-layer chromatography (TLC)

1.9.2 Chromatographic methods

Merck silica gel 60 F_{254} plates (20x20 cm, 0.5 cm thickness) was used for all TLC techniques. The following solvent systems were employed (proportion are v/v), A. n-Butanol : 14N NH_4OH : H_2O (6:1:2), B. CH_3Cl_3 : Methanol (9:1).

The TLC plate was washed by continual development using the same solvent that was used as that during chromatography. This was done in an air tight glass tank; the solvent was poured to a depth of 1 cm. The plate was taken out and allowed to dry at room temperature. The container lid was replaced and the solvent was allowed to equilibrate for 15 minutes.

After spotting with various extracts at the origin CK markers; BAP and K were spotted alongside the extract spots. The plate was then washed in solvent A. The procedure was repeated for solvent B.

1.9.3 Evaluation and identification

After development in the solvents above, time and length of run were noted. The chromatograms were dried in warm air. The plates were then viewed under a UV (254 nm) lamp. Fluorescent spots located under UV as dark spots against a fluorescent background were marked and their colours noted. Photographs of chromatograms were taken under UV light. Identification was also performed by spraying the plate with $AgNO_3$ - Bromophenol blue solution, sometimes called Wood's reagent. This reagent was prepared by dissolving 0.2 gm of bromophenol blue in 50 ml acetone. This was mixed with 50 ml of 2 % aqueous $AgNO_3$.The reagent was kept in a fridge, in a bottle covered with aluminium foil, before use. Cytokinins show up on TLC plates as blue spots after being sprayed with this solution.

2. EXPERIMENTAL STUDIES FOR THE MICROPROPAGATION OF *S. AUCUPARIA*

2.1 Experiments to assess the effects of different combinations and concentrations of plant growth regulators (BAP and IBA) on multiple shoot proliferation in *S. aucuparia*

Experiment 1: The effects of BAP and IBA on excised embryos together with a high percentage of sucrose added to the basal medium

General materials and methods applied in this experiment are specified in Section II. The basal medium used was MS half-strength, 0.6 % Difco agar and 3 % sucrose and this medium is called the **proliferation medium** throughout this study. Nine cytokinin (BAP) concentrations (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1, 2 and 5 mg/l) and ten auxin(IBA) concentrations (0, 0.01, 0.02, 0.04, 0.05, 0.08, 0.1, 0.2, 0.5 and 1 mg/l) were used in every combination, thus giving a total of 90 combinations (9 x 10

diallel). Each combinations was replicated three times. Embryos were prepared in the manner previously described (Section II.2) and were placed on solidified nutrient media in culture vessels (two embryos per vessel). All 270 vessels were randomly placed in a growth cabinet at a constant temperature of 20°C and under 16 hour photoperiod. Observations were made on hypocotyl, root and shoot growth after one and one half months of incubation. Fungal or bacterial infection, swelling and callusing was also noted.

To easily refer to each treatment, was coded (Table.III.2).

Table III.2: Codes for each concentrations of PGR's used in Experiment 1

BAP(mg/l)	Code	IBA(mg/l)	Code
0	a	0	1
0.1	b	0.01	2
0.2	c	0.02	3
0.4	d	0.04	4
0.5	e	0.05	5
0.8	f	0.08	6
1	g	0.1	7
2	h	0.2	8
5	i	0.5	9
		1	10

Experiment 2: Influence of cytokinin and auxin on shoot multiplication and elongation.

Two types of explants were used to investigate the influence of the above plant regulators (see Section II.3.1). The first (is called **Experiment 2a** for easy reference) were 8-month old seedlings to identify combinations that would promoted multiple shoot formation. Nodal segments of 8 month-old seedlings were cut aseptically 2 - 3 mm from the base of the seedling (average height between 20 - 50 mm), each producing 1 - 3 explants of between 15 - 20 mm in length. Each explant had between 1 - 4 leaf-joints, before insertion into the culture medium, the leaf blade at the base of the stem was cut off leaving the petiole intact, also the top leaves were trimmed in half. The explants were cultured in the **proliferation medium** using the nine cytokinin (BAP) and ten auxin (IBA) concentrations (Table III.3). A total of 90 combinations (9 x 10 diallel). Each treatment consisted of 6 explants, 2 explants per petri dish.

The second type of explant (**Experiment 2b**) was two-month old seedlings and refined the results of the **Experiment 2a** to identify the best hormonal combination for shoot proliferation without callus formation.

Nodal explants of 2 month-old aseptically germinated seedlings were cut into 10 - 20 mm lengths and pushed about 2 mm into the **proliferation medium** which consisted six cytokinin (BAP) and four auxin (IBA) concentrations (Table III.4). Each treatment consisted of 10 explants, 2 explants per petri dish (4 cm diameter). Each petri dish was sealed with parafilm. Observations were made after one month on the number of multiple shoots that developed. From the data collected an index of

Table III.3: Concentrations and codes of PGRs used in Experiment 2 employing 8 month-old shoots as the initial explants .

BAP(mg/l)	Code	IBA(mg/l)	Code
0	a	0	1
0.1	b	0.01	2
0.2	c	0.02	3
0.4	d	0.04	4
0.6	e	0.05	5
0.8	f	0.08	6
1	g	0.1	7
2	h	0.2	8
5	i	0.5	9
		1	10

Table III.4: Concentrations and codes of PGRs used in Experiment 2 employing 2 month-old shoots as the initial explants.

BAP(mg/l)	Codes	IBA(mg/l)	Codes
0	a	0	1
0.05	b	0.01	2
0.1	c	0.02	3
0.15	d	0.04	4
0.20	e		
0.30	f		

biomass generated by each treatment was calculated by multiplying the mean shoot length and its corresponding mean number.

2.2. Experiments to investigate the influence of auxins and a hormone rooting powder on rooting formation of excised shoots.

Experiment 1: The influence of IBA in either a high or a low sucrose medium on root formation on excised shoots.

For the rooting treatment, induced shoots from Experiment 2 (Section III.2.1) were incubated in the proliferation medium with differing combinations of IBA (0, 0.01, 0.02, 0.05, 0.10, 0.20, 0.30 and 0.40 mg/l). The excised shoots, between 5 - 10 mm long, were dipped about 2 - 3 mm into the medium. Each vial contained two explants and each treatment was replicated 8 times. The same procedure was repeated with a lower concentration of sucrose (0.8%) in the medium together with six concentrations of IBA (0, 0.01, 0.02, 0.03, 0.04 and 0.05 mg/l), replicated 5 times. Observations were made on root number, root length and the degree of callus formation after two months of incubation. In addition, the shoot height was measured in the second experiment.

Experiment 2: The influence of NAA in high sucrose medium on root formation of excised shoots.

An experiment was conducted using NAA and 3 % sucrose similar to that of Experiment 1 above.

Experiment 3: Influence of combination of auxins on root formation of excised shoots.

Shoots, 10 - 20 mm long derived from shoot proliferation experiments (Section III.2.1, Experiment 2), cut at an oblique angle, planted 3 - 5 mm deep into proliferation media supplemented with 0.8 % sucrose and various combinations of two auxins IBA and NAA (0, 0.01, 0.05, 0.1, 0.2,

0.3, 0.4 and 0.5 mg/l). Two explants were planted into a 60 ml plastic containers into which was previously placed 20 ml of medium. Each treatment was replicated 3 times. Records of the number of roots, root lengths and incidence of callus were taken four months after incubation @ 20°C in Gallenkamp incubators. The occurrence of callus was estimated by measuring their diameter.

2.3 Experiments on hardening off *in vitro* rooted shoots

Experiment 1: The influence of auxins and a hormone rooting powder in rooting formation of excised shoots *in vivo*.

The following experiments investigated the possibility of increasing the efficiency of propagation procedures by directly transplanting treated shoots *in vivo* into rooting medium consisting of an equal mixture of sand and peat. Volumes of sieved + mixed peat and sand, formed the rooting medium. Disposable plastic containers were filled with this medium. Five such containers fitted well into one propagator. The rooting medium was watered with sterile water and excess water allowed to drip into the propagator tray. Excised shoots (obtained from the shoot proliferation experiments), 10 - 15 mm in length, each of which possessed 1 - 3 leaf-joints were prepared aseptically in a laminar flow cabinet. They were then treated with three different types of growth substances i.e. commercial hormone rooting powder (Seradix 3), or IBA or NAA (Table III.5).

The shoot bases (± 3mm) were dipped into freshly prepared auxin solution for about 10 seconds before insertion into the rooting medium. Shoots treated with rooting powder were initially wetted with sterile water at the shoot base before dipping the base into the powder. Any excess rooting powder was removed by lightly tapping the shoot. The

Table III.5 : Concentration of auxins used for in vivo rooting experiment.

Auxins	Concentration (mg/l)			
Indole-butyric acid (IBA)	100	250	500	1000
Naphthalene-acetic acid (NAA)	100	250	500	1000

controls were treated with distilled water. The treatments were performed in a completely randomised manner with three replicates and ten shoots per replicate. Each replicate consisted of two rows of five shoots. The cuttings were incubated in a controlled humidity Fison's Growth Cabinet (model 600 G3/THTL) at 20°C temperature, at 80 % relative humidity and a 16 h photoperiod. Watering were carried out every two days by spraying with sterile water. Data was recorded six weeks after planting. All rooted shoots were carefully lifted out and the number of roots produced per shoots were counted. Non-rooted and dead shoots were also recorded.

Experiment 2: To investigate the hardening off rooted shoots for transfer to soil in the greenhouse.

Rooted shoots produced *in vitro* from **Experiment 2b** (Section III.2.1) were carefully lifted out from the plastic container and all traces of agar removed by rinsing in sterile distilled water. Five such shoots were transplanted into plastic containers with rooting medium (Section III.2.3); five such containers formed the control. The same number of shoots and replicates were also prepared, but roots were treated by dipping in freshly prepared 0.5 % Benomyl solutions for 10 seconds to

prevent fungal infection. All containers were placed randomly in two propagators and the environmental conditions provided were the same to that employed in **Experiment 1** above. The propagators were kept in the greenhouse which was well-lit for 16 h daily, plantlets were watered every other day. After two weeks the top cover of each propagator was removed. The plantlets were allowed to grow until two months after planting after which rooted plants were carefully lifted out and the number of roots produced + their length were measured. The number of dead and infected plantlets were immediately removed and recorded as soon as they were discovered.

Experiment 3: To investigate the effect of *in vivo* rooting of shoots in greenhouse conditions

This experiment was designed to observe the effect of commercial rooting powder on the number of shoots forming roots when shoots were directly planted into the rooting medium (section III.2.3) in greenhouse conditions. Fifty excised shoots produced *in vitro* were treated with Seradix 3 (see **Experiment 1**) and planted directly into the rooting medium of sand and peat. The propagator trays were maintained under the same environmental condition as in **Experiment 2** above. Two weeks acclimitization to the greenhouse conditions was allowed after which the cover of the propagators were removed and subsequently plantlets were watered twice daily with tap water, using a fine rose spraying gun.

The plantlets that survived were left to grow for two months in the greenhouse after which they were potted on individually and left to grow outside in the open. General observations were made on the number of plantlets that died and those that rooted and non-rooted.

SECTION IV

RESULTS AND DISCUSSIONS

1. FLORAL BOTANY AND DEVELOPMENTAL STUDIES

1.1 A preliminary survey of seed production of *Acer platanoides* L. (Norway maple) and *A. pseudoplatanus* L. (sycamore)

The maple tree from which the fruits were sampled was an old fully matured tree at least 40 - 50 years old. It bore fruits heavily, but the fruits were small, typical of fruits from an aged tree. The summary of the data is illustrated in Table IV.1 below.

Table IV.1: A summary of a preliminary survey on seed production of *Acer platanoides* L.(Norway maple) and *A.pseudoplatanus* L.(Sycamore)

Parameter recorded	Maple	Sycamore
(a) Total number of fruits collected	1539	528
(b) Total number of fruits with filled seeds	124	417
(c) Total number of fruits with empty seeds	1415	87
(d) Total number of fruits with bad seeds	19	24
(e) Percentage of filled seeds	8.1 %	79 %
(f) Percentage of empty seeds	91.9 %	16.5
(g) Percentage of bad seeds	1.2 %	4.5 %

In contrast, the sycamore tree, was about 15 -20 years old, a medium size tree, bearing fruit lightly but the fruits were bigger and of apparent good quality.

In the maple, the percentage filled seeds recorded was only 8 %. Abnormal seeds, either rotted or which became black in colour were considered bad seeds and they account for only 1.2 % of the total fruits collected (Table IV.1). In the young sycamore tree, the percentage of filled seeds was very high (79 %) and a very low percentage of empty seeds (16.5 %) was recorded. Bad seeds accounted for 4.5 % of the total.

1.2 A preliminary survey of flowering and fruiting of *S. aucuparia* L. (Rowan or Mountain Ash)

By the 19 - 20th May 1987, flower buds appeared on tree A. Later,on the 1st June , the flower buds from tree B, appeared, they were whitish green in colour. These flower buds were in corymbs and both trees were heavily laden with flower buds. Table IV.2 below summarizes the results of such recording of the reproductive cycle of these trees.

The duration from the bud stage to fruit set was about three months. The opening of flower buds, initially appeared not to be synchronous and the opening of flowers was scattered within a corymb and between corymbs. These temporal differences in flower opening not only occurred within and between corymbs in an individual tree but also, between trees. The small white flowers , numerous and densely packed in a flat-topped inflorescence, had all opened by the end of May or June. The flowers were powerfully scented with a fetid fragrance.Many kinds of insects were attracted by the scent and nectar and thereby pollinated the flowers.

Table IV.2 Summary of the data on flower buds, flower opening, fruit set of *S. aucuparia* L. from Tree A and Tree B, each has 9 and 8 corymbs tagged respectively.

Tree A:

Corymb	Bud No.	Flower No.	Fruit No.	% Fruit Set	% Flower Drop
I.	237	128	0	0	100
II.	140	19	14	10	90
III.	103	20	2	2	98
IV.	252	136	1	0.4	99.6
V.	166	128	11	6.6	93.4
VI.	53	1	0	0	100
VII.	33	13	0	0	100
VIII.	40	38	6	15	85
IX.	37	36	6	16.2	83.8
Mean	118	44.1	4.4	5.8 %	94.4 %

Tree B:

I.	189	55	6	3.2	96.8
II.	49	48	0	0	100
III.	54	15	11	20.4	79
IV.	137	28	6	4.4	95.6
V.	180	174	17	9.4	90.6
VI.	156	141	4	2.6	97.4
VII.	133	128	30	22	78
VIII.	64	52	0	0	100
Mean	120.3	80.2	9.3	7.8%	92.2%

The percentage of flower drop was above 90 % in both trees, A and B. The flowers seemed to open for two or three days after which the styles and stigma turned brown, wilted and shrivelled. The flower soon abscised. Detection of pollen tubes in ovaries of excised flowers proved unsuccessful, making it difficult to estimate which abscised flowers were fertilized. However, the percentage of fruit set was very small, at a mean of 6.8 %.

1.3 Discussion

This preliminary survey aimed to visualize tree seed production of some of the broadleaved tree species of the local flora within the vicinity of Edinburgh city. One of the most important problems associated with collecting tree seed is its irregularity and unpredictablility of flowering and fruiting (Gordon and Rowe, 1982).

Generally, through reconnaissance and seed collecting trips, the year 1987 proved to be a difficult year to find good fruiting on broadleaved trees. There were some scattered fruiting of ash *(Fraxinus excelsior* L.), oak (*Quercus spp.*), lime (*Tilia platyphyllos* L.) and elms (*Ulmus glabra*) that were found along the road sides or opening in a woodland. Many beech (*Fagus sylvatica* L.) and oak (*Quercus spp*) trees were found growing in woodlands near Dalmeny and Dalkeith but none of them possessed fruit. However, in the case of Norway maple, rowan and sycamore, fruiting trees were easily found in abundance.

The individual maple tree studied, represented an example of a fully mature tree with heavy fruiting, but producing only 8 % filled seeds and of unknown quality. Ninety-two percent of the fruits were empty. This seems to be the norm in many tree species, that seed production decreased as it gets older, although sometimes such trees will produce a big crop of small fruits with sterile seeds. Fertilisation, thus does not guarantee seed formation as the process can be halted by adverse conditions and predation, at almost any time up to full maturity (Gordon and Rowe, 1982).

However, in contrast, the young Sycamore tree studied , which had light fruiting, produced a high percentage (79 %) of good seeds and only a small percentage (16.5 %) of empty seeds.

These preliminary observations on the flowering and fruiting behaviour of rowan *(S. aucuparia* L.), have shown that, the timing of flower opening could vary within a corymb,and between corymbs in the same tree and between different trees of the same species. Low temperature markedly delayed and prolonged the time taken for buds to open (Matthews, 1963). Once the flower opened it emits a scent that attracted potential pollinators, which, more often than not ,destroyed other neighbouring flowers during their visits, thus in itself promoting flower drop . The receptive period of an individual stigma also varies greatly and this is closely related to the opening of the flower. If pollination is successful, it will lead to fertilisation of the ovule forming a zygote and later forming a fruit. However, if no compatible pollen falls within the receptive period, the flower will drop. The attempt to detect fertilised flowers by staining the pollen tubes of the flower that had dropped failed. Most of the suspected fertilised flowers, judging by the development in size of their carpel, had no detectable pollen tubes. The trees under study were probably hybrids.It was reported (Gordon and Rowe, 1982) that hybrids may develop seed without any fertilisation taking place (apomixis) and the genus *Sorbus* is more or less completely apomictic. Seeds developed by apomixis have progeny resembling their mother plants.

Some pollinated flowers were predated by the larvae of *Torymus druparum* (Gordon and Rowe, 1982) which also feed on the fruits burrowing

into the seeds. Finally, the birds which frequented the trees fed on the fruits and hence reduced the size of the seed crop.

The percentage of flower drop in rowan trees that grew in the semi-open woodlands and along paths were found to be very high and the percentage seed set was unexpectedly small. A more detailed study appears to be needed to further unravel the flowering and fruiting behaviour of rowan.

2. TESTS OF SEED VIABILITY

During the preparation of the seeds for the biochemical and excised embryo test the number of rowan seeds that were filled, empty, insect damaged or black were noted. The percentage of filled seeds was 64.67±0.9 % and the empty seeds, insect damaged and black coloured seeds were 21.46±2.0, 2.21±1.29 and 11.66±1.43 % respectively.

The viability of the filled seeds of rowan after evaluation by tetrazolium test (Plate IV.1) was 61.7 % whilst the excised-embryo test gave an estimated percentage viablity of 73.3 %. Whereas the viability of the sycamore seeds obtained from tetrazolium test and excised-embryo test were 72 and 87.5 % respectively. Lime gave a 83.6 % figure with the Tetrazolium Test and the excised embryo test on them was discontinued due to difficulties in excising the seed coats without injuring the embryos.

Plate IV.1 : Tetrazolium staining of Sorbus aucuparia L. embryos. The embryos with a (+) ve sign were considered viable, a (-) ve sign considered not viable.

3. BREAKING OF SEED DORMANCY IN *SORBUS AUCUPARIA* L.

3.1 The effects of stratification at different temperatures (Experiment 1, 2, and 3)

The results of the Experiment 1, 2 and 3 are included in the summary table (Table IV.3) for comparative purposes with other results from Section IV.3.1 - 3.2.

Sorbus aucuparia L seeds germinated progressively after 5 weeks incubation at 1°C, after 24 weeks 50 % of seed had germinated (Fig IV.1a). Intensive Germination Period (IGP) started about the 9th week and lasted until the 18th week. Therefore the duration of IGP was 9 weeks and the Median Germination Week (MGW) occurred about the 14thweek of incubation.

With fresh seeds collected from a single tree and immediately stratified at 4°C , the duration of IGP was reduced to 5 weeks and more remarkably, Total Germination Percentage (TG) was enhanced to 90 % and MGW occurred just after the 13thweek of incubation (Fig.IV.1b).

There was no significant difference between stratification at 1°C and 4°C (Fig.IV.1a,c and d) and between sterilised and unsterilised seeds prior to incubation at 4°C (Fig.IV.1c and d). Total germination obtained of all seed lots for 1°C stratification was 53%, a little lower than those obtained for sterilised and unsterilised seeds at 4°C, which was 66% and 63% respectively. Unsterilised seeds had shorter IGP (12 weeks), in contrast to the sterilised seeds which had an extended IGP (16 weeks)(Table IV.3, Fig IV.1c and d). Seed incubated at 1°C took 7 weeks to complete IGP (Table IV.3, Fig IV.1a)as compared to 12 - 16 weeks for the 4°C treatment (Table IV.3, Fig IV.1c and d).

Table IV.3 : Summary of results of breaking of dormancy of Sorbus aucuparia L. Seed by protracted cold temperature or combination of warm–cold stratification temperature

Source	Treatment	IGP (weeks) Began	IGP (weeks) Ended	Duration of IGP (weeks)	MGW	TG%	(SED)	Figures
Seedmen[1]	1°C	9.5	16.5	7	12.5	53	(0.6)	IV.1a
EUC (fresh)[2]	4°C	11	16	5	13.5	90	(3.0)	IV.1b
Seedmen (sterilised)	4°C	10	26	16	15	66	(0.7)	IV.1c
Seedmen (unsterilised)	4°C	9	21	21	13	63	(1.3)	IV.1d
Seedmen	6 m.[5] 10°C/4°C	15	19	4	17	90	(1.2)	IV.2a
Seedmen	6 m. 15°C/4°C	14.5	20	5.5	17.5	72	(1.0)	IV.2b
Seedmen	6 m. 20°C/4°C	8	11	3	9.5	84	(1.9)	IV.2c
EUC (fresh)	2 w.[4] 20°C/4°C	14.5	22	7.5	18	98	(0.3)	IV.3
Seedmen	1 m. 1°C/3GT[3]/4°C	16.5	21.75	5.25	18.5	86	(4.7)	IV.4a
Seedmen	2 m. 1°C/2GT/4°C	16.25	21	4.75	17.25	70	(3.4)	IV.4b
Seedmen	1 m. 1°C/GT/1 m. 4°C/GT/4°C	12.5	22	9.5	17	94	(1.7)	IV.5a
Seedmen	2 m. 4°C/GT/2 m. 10°C/4°C	15.25	25.5	10.25	20.25	66	(2.3)	IV.5b

1. Seedmen – Seeds obtained from British Seed Houses Ltd.
2. EUC – Edinburgh University Campus (Gallenkamp incubator)
3. GT – Germination Test at 20°C in continuous light
4. w – week
5. m. – month/months

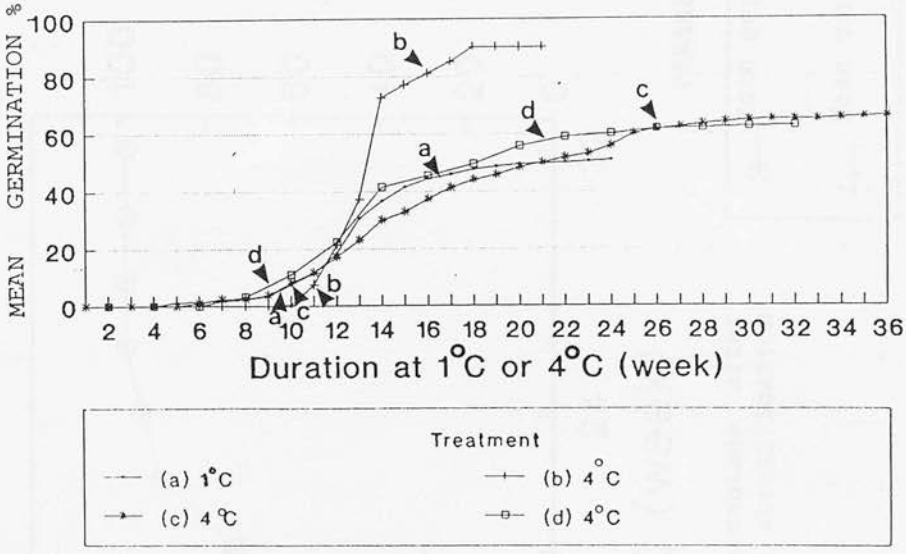


Fig. IV.1 : Mean cumulative germination percentages of Sorbus aucuparia L. seed at 1°C and 4°C stratification temperatures.

➤ An arrow followed by a letter denotes the ending and beginning of IGP for each treatment

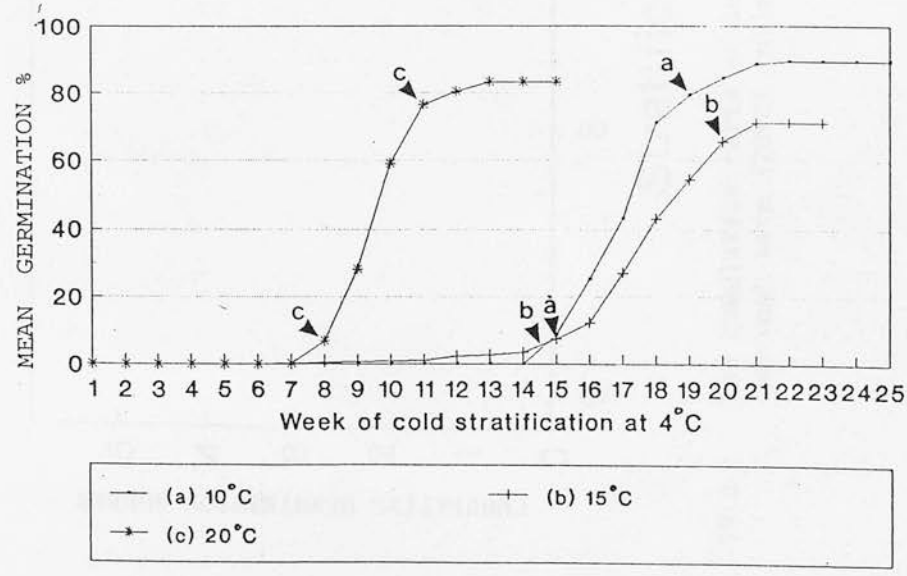


Fig. IV.2 : The cumulative curve of seed germination after 6 months either at 10°C, 15°C or 20°C followed by cold stratification (4°C)

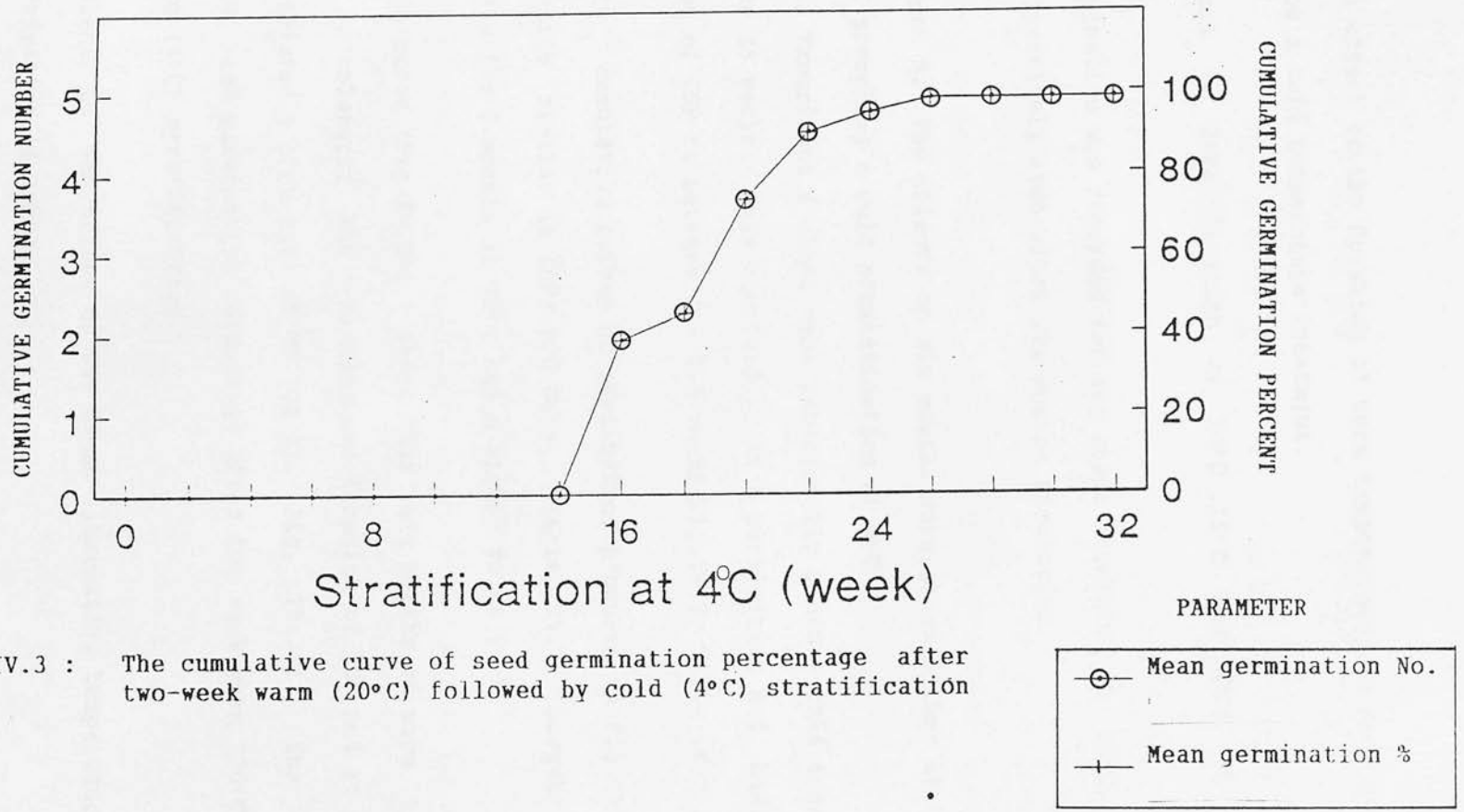


Fig. IV.3 : The cumulative curve of seed germination percentage after two-week warm (20°C) followed by cold (4°C) stratification

3.2 The effect of the duration of warm temperatures on seed germination preceding a cold temperature treatment.

Experiment 4: Stratification at 10°C ,15°C and 20°C at different periods.

No germination was recorded for any seeds incubated at 10°C, 15°C or 20°C respectively even after six months incubation.

Experiment 5: The effects of six months warm phase either at 10°C, 15°C or 20°C preceding a cold stratification at 4°C

Seeds in **Experiment** 4 above were subsequently given a cold treatment at 4°C for 25 weeks. This resulted in 80 % germination and hastened the duration of IGP to between 3 - 5.5 weeks(Fig.IV.2, Table IV.3).

The cumulative curves of germination produced in Fig.IV.2a and b were quite similar in IGPs and MGWs, (Table IV.3) however , seeds incubated for 6 months at 10°C had a higher TG of 90 %.

The curve (Fig.IV.2c), shows that six months of warm temperature (20°C) accelerated the beginning and duration of IGP and at the same time achieved a high rate of TG (84 %). Fig. IV.3 : The cumulation curve of seed germination percentage after two week warm (20°C) followed by cold (4°C) stratification.

Experiment 6: The effect of two weeks alternating temperature on the after-ripening of rowan.

A shorter warm phase (20°C) of two weeks (Fig.IV.3) preceding cold stratification gave an even better TG(%), at the same time maintaining the synchrony of germination(TableIV.3)

3.3 The effects of various combinations of alternating temperatures.

Experiment 7: The effects of various combinations of warm and cold preceding stratification at 4°C

Generally all treatments accelerated IGPs with very high TGs (Figs.IV.4a & b, IV.5a & b and IV.6). Within the tested alternating temperature conditions, there seems to be no particular combination or duration of alternating temperatures that was better than any other treatment.

3.4 Discussion

It has been known for a long time that seed dormancy in most temperate species can be broken by cold stratification (Flemion, 1931; Nikolaeva, 1977). The normal techniques for propagating woody ornamentals from seed begins with sowing in the autumn, facilitating a period of winter stratification to break dormancy, so that germination occurs in the spring (Gordon and Rowe, 1982). The present study has confirmed this phenomenon. The design of the experiments were similar, repeated for different sources of seeds, either fresh or acquired from British Seed Houses Ltd.. Their performance after dormancy breaking by stratification at different temperatures were compared. Stratification at 1 and 4°C were effective and the seed germinated in the cold (at 1°C). The slow chitting of seeds during stratification is wasteful, since these seeds are the least dormant and yet they germinated out of step with the majority of seeds, and unless these are taken out and grown on, which is labour intensive, this early chitting should be avoided. The best technique is to achieve an early start to IGP, of the shortest duration and maximizing the highest total germination. A summary of the treatments and their performance can be compared (Table IV.3). The

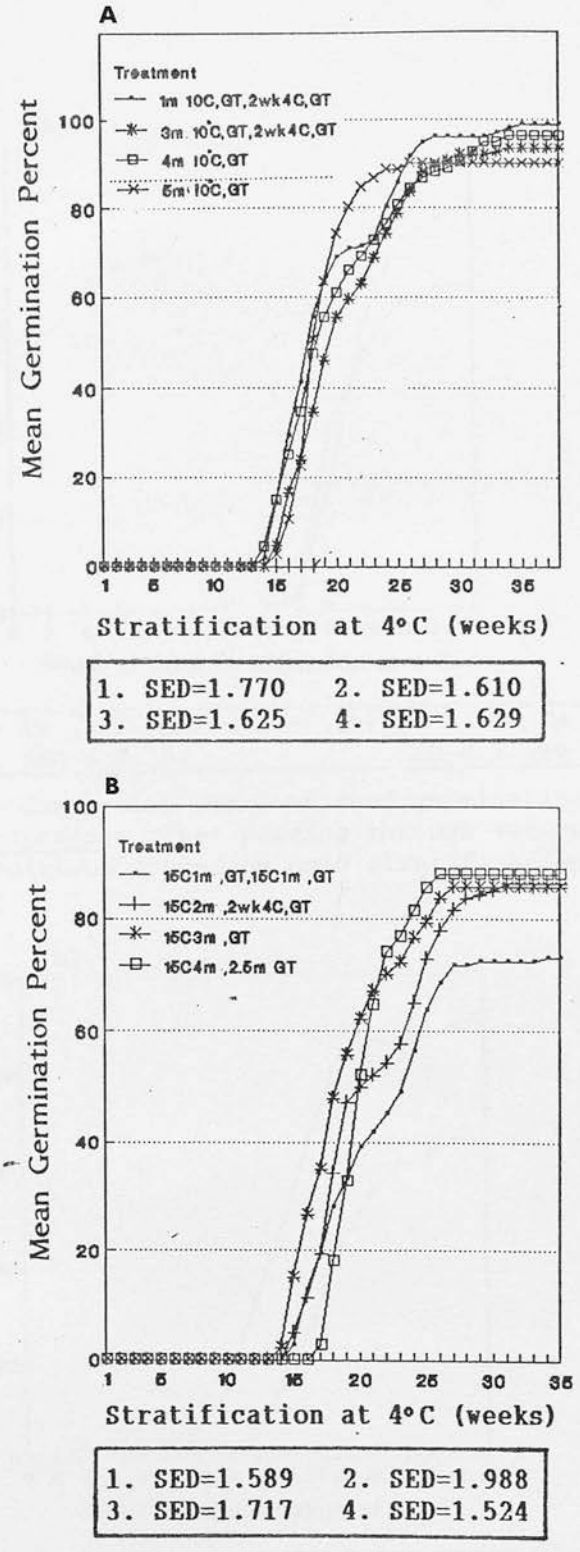


Fig. IV.4: The cumulative curve of seed germination of *Sorbus aucuparia* L. after various treatments preceding 4°C stratification

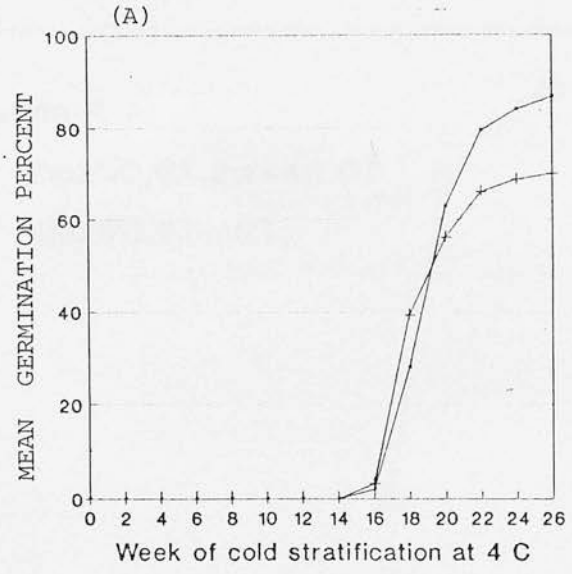
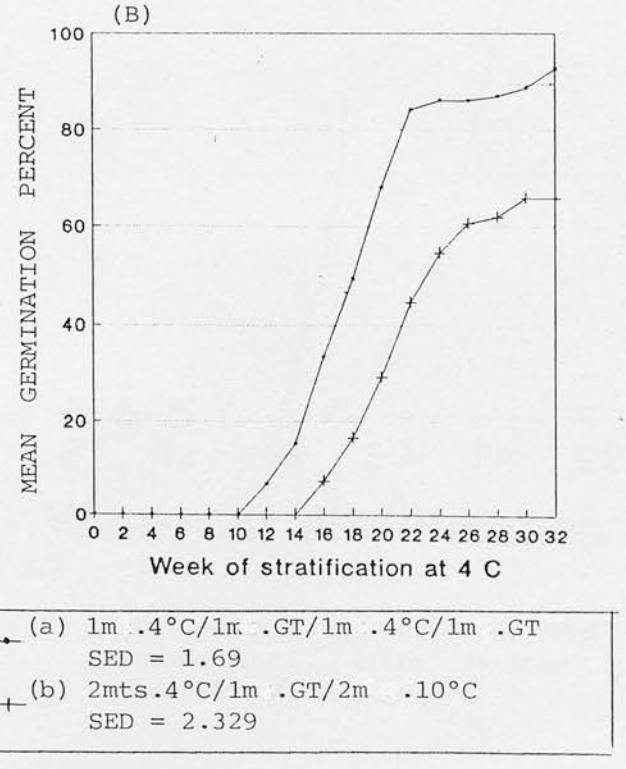


Fig. IV.5 : The cumulative curve of seed germination of Sorbus aucuparia L.after passing through various temperature conditions preceding cold stratification at 4°C



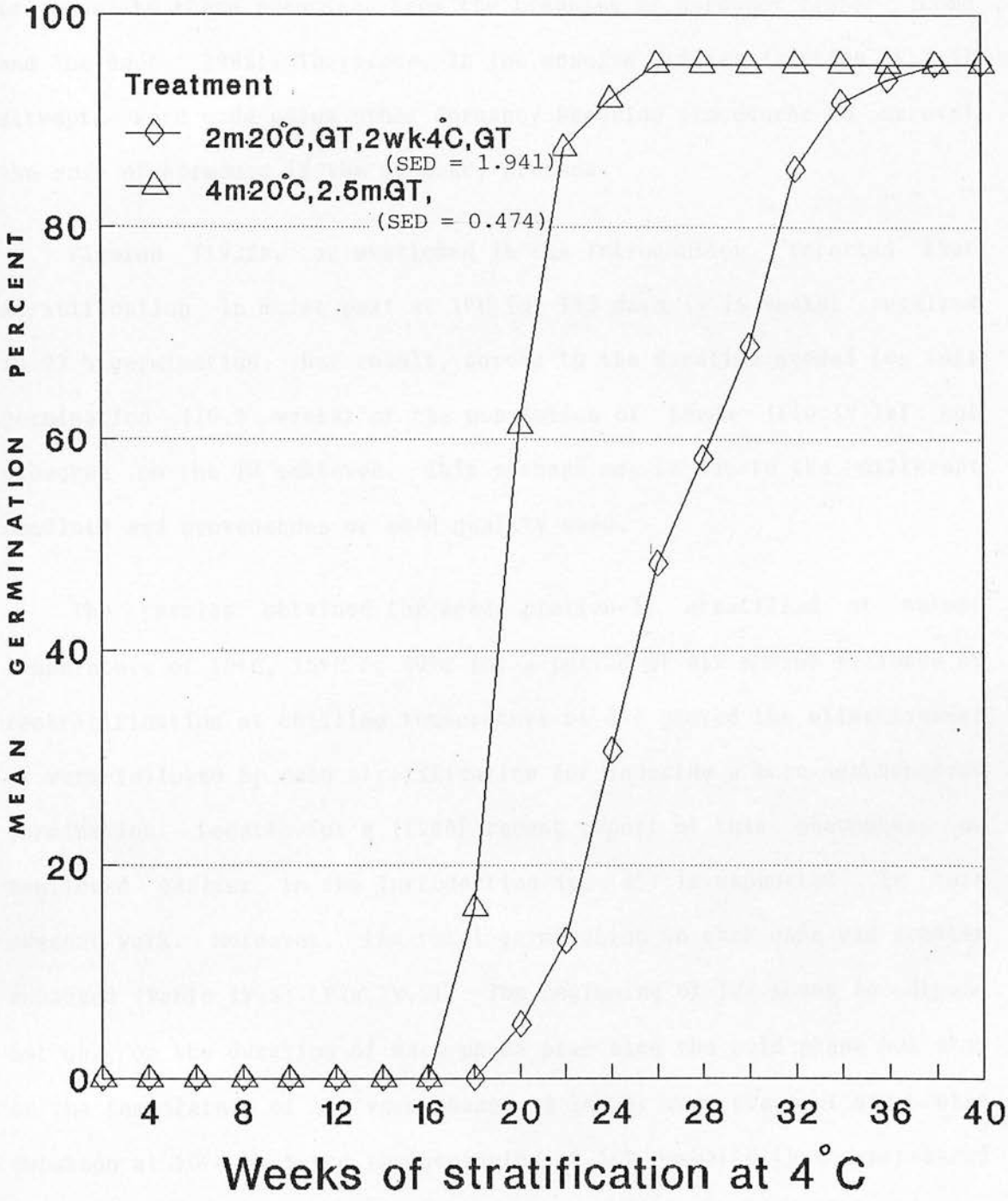


Fig. IV.6 : The cumulative curve of seed germination of *Sorbu aucuparia* L. after various treatments preceding 4° stratification.

problem in studying the process of dormancy breaking by cold stratification at the temperature used, is that germination and growth may occur during the cold treatment, in which case it will be impossible to separate these phenomena from the breaking of dormancy proper (Comé and Thevenot, 1982). Therefore, in the ensuing studies (section IV.3.4) attempts were made using other dormancy breaking procedures to unravel the role of hormones in the dormancy process.

Flemion (1931), as mentioned in the Introduction, reported that stratification in moist peat at 1°C for 113 days (± 16 weeks) resulted in 93 % germination. Her result, agrees to the duration needed for full germination (16.5 weeks) of the population of seeds (Fig.IV.1a) but disagree on the TG achieved. This perhaps may be due to the different seedlots and provenances or seed quality used.

The results obtained for seeds previously stratified at warmer temperature of 10°C, 15°C or 20°C for a period of six months followed by restratification at chilling temperature of 4°C proved the effectiveness of warm-followed by cold stratification for inducing a more synchronized germination. Lenartowicz's (1988) recent report of this phenomena, as mentioned earlier in the Introduction (p. 45) is supported in this present work. Moreover, the total germination in each case was greatly enhanced (Table IV.3; Fig.IV.2). The beginning of IGP seems to depend not only on the duration of warm phase preceding the cold phase but also on the temperature of the warm phase. A longer warm phase of six months duration at 20°C hastened the beginning of IGP compared that experienced after only 2 weeks warm followed by the cold phase, which took a longer time to reach IGP (Table IV.4, Fig.IV.2).

The effect of various combinations of alternating temperatures of warm and cold, preceding stratification at 4°C, showed that the temperature of the warm phase (10°C,15°C and 20°C) had little effect on seed germination during the cold phase. Perhaps, the duration of the warmer temperatures could be manipulated to produce the same effect. Longer time at 10°C preceeding the cold phase (Fig.IV.5), effected a similar germination curve on seeds exposed to less time at 20°C (less GT exposure). However, in this study all the seed tested had at least experienced a warm GT temperature of 20°C, which was perhaps the effective warm temperature that accelerated IGPs with very high TGs. Lenartowicz (1988) who studied the effect of of the warm (15°C - 25°C) and cold (1°C - 5°C) temperature on rowan had found that there was no significant effect of the temperature of the warm phase either on total germination %, or on the beginning or duration of IGP. However, 25°C acting for 1 or 2 weeks accelerated MGW more than 20°C or 15°C lasting for the same time.

Sterilisation of seeds had no effect on total germination, however, it shortened IGP considerably. It is reasonable to argue that the low TG was due to the prolonged stratification at 4°C which encouraged fungal and viral infections which was favoured by the high humidity in the petri dishes. Infections and the fungal exudates damaged the germinating seeds and reduced the quality of the seedlings produced and increased the mortality rate of the seeds.

Fresh seeds, normally possess a high percentage of viable and vigorous progeny , which produced the highest percentage germination, though their IGP duration was delayed, but it was the shortest in

duration. This was advantageous in inducing a synchronized germination to the population of the freshly *S. aucuparia* seeds. But the effect of two week warm period followed by cold stratification on the fresh seeds was dramatic, producing 98 % germination. The same effect has also been reported for *Prunus avium* seeds (Suszka (1962).

Most reports recommended that a low temperature stratification gives the best results in breaking the dormancy of rowan. This includes the routine testing required by ISTA (ISTA, 1985). There have been only two reports (Gordon and Rowe, 1982; Lenartowicz, 1987) recommending for *Sorbus aucuparia* L. seeds to be treated by a two week warm followed by a cold phase to enhance dormancy breaking. The results of the present study confirm these recommendations.

3.5 The effect of stratification at 4°C on different media.

3.5.1 The germination percentage of seed following stratification (Experiment 8)

(a) At four weeks stratification No germination was recorded in all the seed samples, in all the media tested, even after incubation for up to 28 days at 20°C and continuous light.

(b) At six weeks stratification

After six weeks of stratification 16 % of the seeds germinated in vermiculite , 4 % in sand and 2 % in both the polythene bag and peat medium (Table IV.4). The remainder of the seeds remained fresh , but ungerminated.

Seeds germinated best in either vermiculite or sand (24 %) followed by peat (18 %) and polythene bag (8 %, Table IV.4). The remainder remained fresh, ungerminated.

(d) At ten weeks of stratification

After ten weeks of stratification in the four media, the percentage of germination had risen dramatically to an average of about 80 %. while the remainder remained fresh and unchanged.

The highest germination resulted from the sand medium (88 %) followed by polythene (76 %), peat (74 %) and vermiculite (70 %) (Table IV.4). All seedlings had normal radicle, hypocotyl and shoot extension with the exception of some seedlings stratified in polythene bags which turned brown at the root tips.

Table IV.4 : Percentage germination of *S. aucuparia* seeds after stratification at 4°C in four different media followed by 28 d incubation period in continuous light.

Stratification (weeks)	Sand	Vermiculite	Peat	Polybag
4	0	0	0	0
6	4 ± 8.4	16 ± 12.6	2 ± 6.3	2 ± 6.3
8	26 ± 18.9	26 ± 23.2	18 ± 19.9	8 ± 10.3
10	88 ± 21.5	70 ± 30.2	76 ± 22.7	74 ± 30.2

± Standard deviation , LSD = 10.79 , df = 108.

Mean germination oercentage followed by the same letter are not significant at 0.05 level of significance

(e) At 12 weeks of stratification

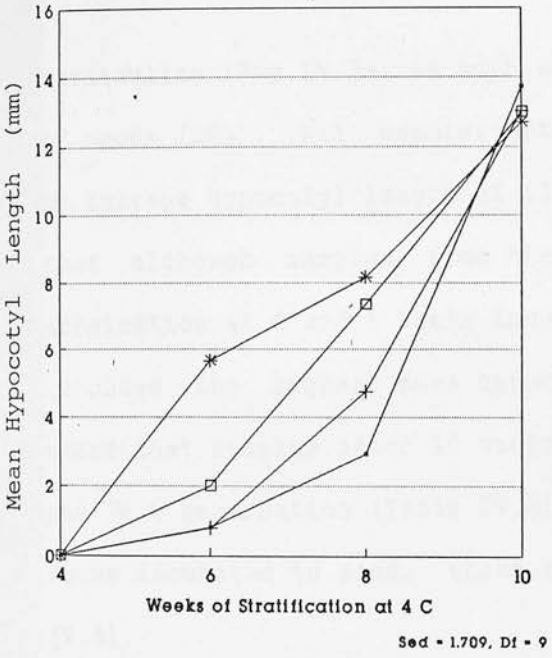
Most of the seeds in all media had chitted by the 12th week.

Analysis of variance of mean germination percentages of rowan seeds after 6, 8 and 10 weeks of stratification at 4°C in four media indicated that there were significance differences ($P < 0.0001$) between weeks but not between media at 0.05 level of significance (Table IV.4). Overall highest percentage of germination was obtained in the order of sand > vermiculite > peat > polythene bag (Table IV.4). There were no significant difference in mean germination of seeds stratified in either sand, vermiculite or peat, and those stratified in peat or polythene bag at $P < 0.05$ level of significance. However, mean germination percentage of seeds stratified in polythene bag was significantly lower than those of sand and vermiculite ($P < 0.05$).

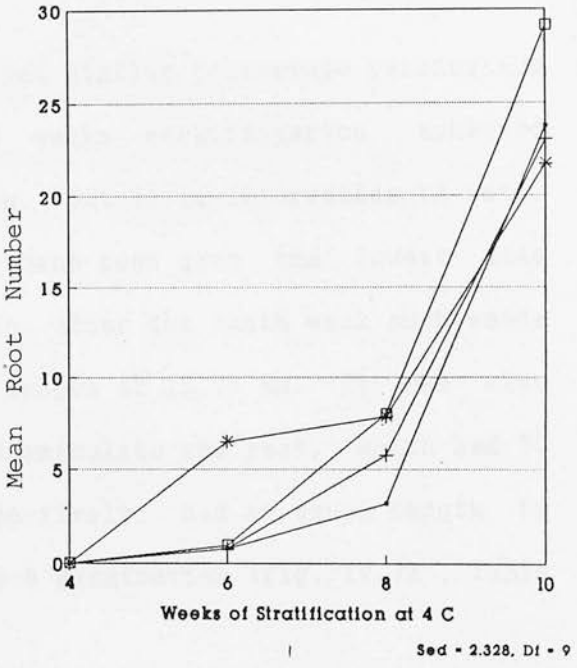
3.5.2 Growth of seedling hypocotyl, root, shoot and leaf number

(i) Mean hypocotyl length (Fig.IV.7a)

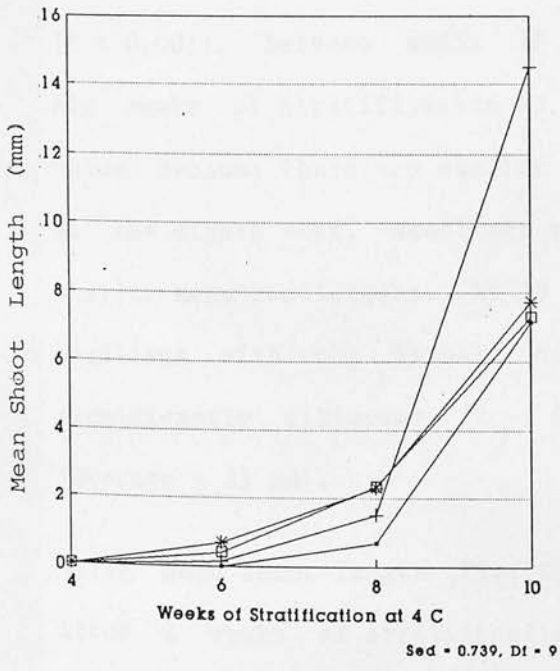
As the period of stratification increased from 4, 6, 8 and 10 weeks in all media, generally there was a progressive and gradual lengthening of the hypocotyl (Fig.IV.7a). There were significant differences ($P < 0.1$) in the mean hypocotyl length between weeks. Though interaction between media and week was not significant. In vermiculite the hypocotyl length of seeds after 6 weeks incubation had tripled compared to that (2 mm) recorded for sand (Fig.IV.7a), simply because there were more with earlier germinated seeds in vermiculite. Whereas those stratified in polythene bags and peat had a similar mean hypocotyl growth of 0.80 mm after six weeks. After 8 weeks, the mean hypocotyl length recorded for samples in sand was not significantly different to that in



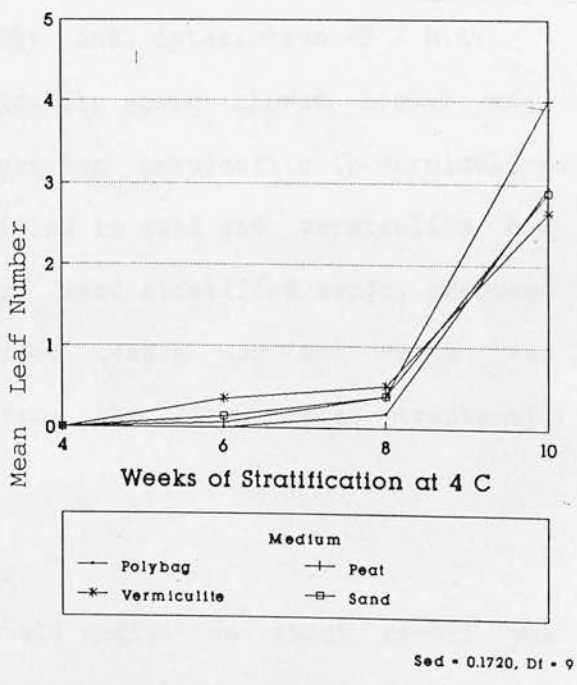
(a) Mean Hypocotyl Length



(b) Mean Root Length



(c) Mean Shoot Length



(d) Mean Leaf Number

Fig. IV.7 : Effects of 4, 6, 8 and 10 weeks of stratification at 4°C in four different media followed by 28 days of incubation (20°C and continuous light) on the growth of seedlings of *Sorbus aucuparia* L.

vermiculite (Fig.IV.7a),as both media had similar percentage germination of seeds (26%). All samples after 10 weeks stratification achieved an average hypocotyl length of 13.12 mm. But it is interesting to note, that although samples from the polythene bags gave the lowest mean germination at 6 and 8 weeks incubation, after the tenth week such seeds recorded the highest mean hypocotyl length of 13.73 mm. It was also noted that samples after 10 weeks in vermiculite and peat, which had 70 and 76 % germination (Table IV.4) respectively, had an equal length to those incubated in sand, which had 88 % germination (Fig. IV.7a , Table IV.4).

(ii) **Mean Rootlength** (Fig.IV.7b)

The mean root length showed significant difference between weeks ($P < 0.001$), between media ($P < 0.05$) and interaction ($P < 0.1$). Six weeks of stratification in vermiculite again showed higher mean values because there was earlier and greater germination in vermiculite. At the eighth week, seedlings stratified in sand and vermiculite had similar mean rootlengths. At 10 weeks, sand stratified seeds, produced seedlings with the highest mean root length (29 mm) which was significantly different ($P < 0.1$) from the other three treatments (average ± 23 mm).

(iii) **Mean shoot length** (Fig. IV.7c).

After 4 weeks of stratification in all media, no shoot growth was recorded. Shoots began to appear after six weeks from seeds stratified in sand, peat and vermiculite, but the growth was very slow and the mean height achieved was less than a millimetre. Whereas seeds that were stratified in polythene bags recorded shoot growth after 8 weeks of stratification.

A trend of inhibited shoot growth was seen after 6 and 8 weeks stratification period. There were no significant difference ($P < 0.001$) in the mean shoot height recorded between the 4^{th} and 8^{th} week and between media. This was followed by an abrupt and significant rise in the mean height after a 10 week period for all samples, particularly, in the peat stratified seeds which had almost doubled (mean shoot length 14.5 mm) the shoot height compared to that achieved by the other three treatments (average 7.38 mm).

(iv) Mean Leaf Number (Fig. IV.7d)

The analysis of variance indicated that the different media and weeks of stratification had a significant effect ($P \leq 0.001$) on the mean leaf number produced by the seedlings after 28 days of incubation.

A leaf was considered and counted based on the shape and its green colour. At 6 and 8 weeks stratification, generally, the seedlings from all the media possessed a rudimentary leaf. However, samples from the 10 week period of stratification, in all media tested, had significantly more leaves formed and their mean leaf number was 3.12 . Ten weeks stratification in peat, produced significantly higher number of leaves (mean leaf number = 4) than those stratified in the other media.

3.5.3 Excised-embryo test and growth of hypocotyls, roots and shoots of seeds that remained ungerminated after 12 weeks of stratification in polythene bag at 4°C (Experiment 9)

The results of GT on excised embryos and their mean hypocotyl, root and shoot growth of the seeds previously cold-stratified in polythene bag for 12 weeks was summarised (Table IV.5). Some of the embryos germinated

Table IV.5 The mean growth of hypocotyl, root and shoot , the percentage of germination and cotyledon greening of embryos after GT. Embryos were excised from seeds that has been cold-stratified for 12 weeks in polythene bag.

Parameter	Germination Test Condition	
	7 day	28d
Mean hypocotyl length (mm)	3.84 ± 0.89	7.54 ± 1.29
Mean root length (mm)	3.14 ± 0.69	5.56 ± 0.89
Mean shoot length (mm)	0	1.66 ± 0.44
Percentage germination (%)	2	62
Both cotyledons fully green (%)	40	24
Semi-greening of one + one cotyledon unchanged (%)	2	2
One cotyledon fully green (%) + one cotyledon unchanged	8	6
One cotyledon fully green (%) + one cotyledon semi-green	0	4
Both cotyledons semi-green (%)	2	2
Both cotyledons unchanged (%)	6	0

at once and produced normal seedlings, others exhibited spreading or greening and growth of one or more of the cotyledons, and still others remained firm and white or creamy and slightly enlarged.

After 7 days at 20°C and continuous light, 42 % of the embryos germinated producing hypocotyl and roots with a mean of 3.84 and 3.14 mm respectively (Table IV.5). No shoot growth was recorded. Embryos that did not germinate, however, showed various combination of cotyledon greening and some remained unchanged (Table IV.5). After 28 days, as

expected all parameters of growth that were measured (Table IV.5) showed a significant increase ($P < 0.001$, by paired-t tests comparison with 50 degrees of freedom). Percentage that did not germinate after 28 day period of GT was obtained by summing all embryos that exhibited various combination of greening of cotyledons i.e. a total of 38 % (24 + 2 + 6 + 2 + 4). There was no embryo that remained totally white /cream or unchanged at the end of the experiment.

3.5.4 The growth of seedlings that germinated in the media from seeds previously cold stratified for 12 weeks.

The results of 28 days incubation at 20°C in continuous light of seedlings that had germinated earlier in each medium after 12 weeks of stratification, is illustrated in Table IV.6 below.

Table:IV.6 Effect of incubation at 20°C in continuous light for 28 days, on 12 week stratified seeds that had germinated in the cold.

	Stratification Media				
Parameter(mm)	Polythene	Peat	Vermiculite	Sand	SED
Mean root Length	17.72	7.31	19.41	25.50	1.638 ***
Mean hypocotyl length	24.06	11.88	27.52	31.38	1.935 ***
Mean shoot length	3.16	3.74	2.49	3.00	0.850 NS

*** very significant ($P < 0.001$)

NS not significant

It should be noted that 5 out of 10 replicates were infected by a fungus that grew in the peat medium.

The analysis of variance indicated that there was a significant difference ($P < 0.001$) in the mean root length and mean hypocotyl length obtained between the four media.

The mean rootlength (25.5 mm) for sand stratified seed was higher than those of the other three media.There was no significant difference between means obtained from vermiculite and in the polythene bag. However, mean root length for peat stratified seedlings was significantly lower (7.31 mm) than that achieved by seedlings on the other three media studied.

Similarly, the highest mean hypocotyl length (31.39mm) was measured in seedlings obtained from sand (Table IV.6), which was significantly different ($P < 0.001$) from the other three media. Mean hypocotyl length of vermiculite stratified seedlings were longer ($P < 0.001$) compared to that of seedlings from polythene or peat. Peat, again was shown to produce seedlings with a poor mean hypocotyl length and was significantly lower than the mean hypocotyl length produced by the other media.

There was no difference in shoot growth of seedlings grown in the different media.

3.5.5 The results of random sampling of 200 seeds or seedlings following cold stratification for 12 weeks.

From the sampling which was performed randomly for each media, the percentage of germinated seeds in the cold after 12 weeks stratification are shown in Table IV.7.

Table IV.7 : Percentage of germinated seedlings from 200 random sampling of seeds and/seedlings, from each medium after 12 and 14 weeks of stratification at 4°C

Medium	Weeks of stratification	
	12	14
sand	38	37
peat	25	12
vermiculite	24	26
polythene bag	14	-

Seeds stratified for 12 and 14 weeks in sand, had a higher percentage germination compared to those stratified for the same period in the other media (Table IV.7). Seedlings obtained from sand medium, were healthier, with good root and root-hair production (Plate IV.2a), whereas those obtained from peat (Plate IV.2b) had some roots infected by a fungus. Vermiculite stratified samples (Plate IV.2c) had very high percentage of dead seeds and most of the seedcoats were almost black in colour. Polythene stratified seed, however, had the lowest percentage of germination.This may have been due to anaerobic condition in the polythene bags. This was observed , as the inner sides of the bag were almost glued to each other, trapping some air and yellowish-coloured pocket of water which was probably stagnated metabolic by-products of the seeds which had chitted. The germinated seedlings (Plate IV.2d) counted was 14 % and the roots suffered signs of scorching and were rust coloured.

Plate IV.2a : Seedlings from sand stratified seeds

Plate IV.2b : Seedlings from peat stratified seeds

Plate IV.2 : Seedlings performance after incubation for 28 days, preceded by the stratification of seed for 8 weeks at 4°C in medium, (a) peat, (b) vermiculite, (c) polythene bag and (d) sand

Plate IV.2c : Seedlings from vermiculite stratified seeds

Plate IV.2d : Seedlings from polythene bag stratified seeds

Plate IV.3a : Seedlings from peat stratified seeds

Plate IV.3b : Seedlings from vermiculite stratified seeds

Plate IV.3 : Seedlings performance after incubation for 28 days preceded by the stratification of seed for 8 weeks at 4°C in medium (a) peat, and (b) vermiculite.

3.5.6 Discussion

Seeds stratified for 6, 8 and 10 weeks in all media studied have showed different germination responses after incubation for four weeks at 20°C in continuous light. Generally, growth of hypocotyls, roots, shoots and the number of leaves was less after 6 weeks of stratification (Fig IV.2a, b, c & d) than after eight weeks incubation (Fig. IV.2a, b, c & d). Those seeds that were released from dormancy after 6 or 8 weeks protracted chilling,regardless of the germination medium grew normally (Plate IV.3a and b) and there were no difference in performance. Nonetheless, it was found that after six or eight weeks of stratification, vermiculite promoted more germination (16 and 26 %, respectively) than the other media. By the tenth week stratification at 4°C, a very high percentage of the seeds had lost dormancy and germinated, producing normal seedlings with good root, hypocotyl and shoot growth and with a high leaf number.

Therefore, firstly,seeds respond differently depending on what media they are germinated on. Secondly, some seeds have a shallower dormancy than others and germinate earlier, compared to the majority of seeds which need 10 weeks (2.5 months) stratification to break dormancy. The medium could play a promotory role in improving the germinative capacity of the seeds and consequent seedling performance. The lengthening of roots, seems best achieved in sand,whereas for shoot growth and leaf production,peat was the best medium. The results of these experiments, suggest that optimum growth of seedlings, could be achieved,through moist stratification, using mixtures of sand and peat. This result is different to that of Flemion (1931) who found that

peat/moss was the best stratification medium favouring higher germination rate compared to that of sand and manure.

Stratification in polythene bag was the simplest method and *S. aucuparia* L. seeds could be easily stratified in bags, while still obtaining a respectable germination rate. But extra care should be taken to ensure enough moisture and good aeration , so that, there is less accumulation of respiratory and metabolic by-products such as CO_2 that upon accumulation will form acidic conditions. This acidic environment, coupled with the heat buildup in the bag could scorch the radicles,giving it a rust-like colouration, which would be detrimental to seedling growth. This was what happened to some of the seedlings, stratified in the polythene bags for a lengthy period, such as 10 weeks. These conditions resulted in lower mean rootlength values (Fig IV.2b), even though the number that germinated was very high (74 %,Table IV.4).

Investigation by the excised embryo method on the viability of ungerminated seeds that had been prechilled (4°C) for 12 weeks in polythene bag (Experiment 9, Section IV.3.3.1) showed all the seeds were viable though with varying degrees of vigour as manifested by their degree of greening of cotyledons. By observing the behaviour and condition of these embryos daily an accurate viability determination could be secured within few days to two weeks (Heit, 1955). All seeds used in this experiment indicated that they were viable and further after-ripening treatment should overcome the deeper dormancy that they possessed. If they were dead,the embryos would become moulded and deteriorate or exhibit brown or black discoloration (Heit, 1955). The main purpose for this test was to check whether lengthy stratification

in polythene bags deteriorated the seeds. Though percentage germination after 28 days at GT was 62 %, however, the mean hypocotyl, root and shoot elongation of the excised embryos from the 12 week batch showed a great difference when compared to the mean values obtained from the 10 week (Fig IV.2a, b and c; section IV.3.3.2). This poor performance of hypocotyl, root and shoot growth in seeds stratified in polythene bag after 12 weeks of cold-stratification in this experiment must be due to poor aeration. Lengthy periods in a relatively small polythene bag, even with frequent aeration alone was not adequate to produce a healthy condition for seed statification. It is suggested that bigger polythene bags be used for better air circulation and for long periods of stratification frequent changes of polythene bag are neccessary to remove metabolic by-products that readily adhered to the inner layer of the bag.

The results of counting the seedlings that germinated after 12 and 14 weeks of stratification (Table IV.7) in each medium showed that sand was the best medium . Sand gave better aeration and allowed for drainage of by-products. Peat, however, due to its better retention of moisture and poor drainage, coupled with heat buildup due to prolonged stratification of respiring seeds provided ideal conditions for the growth of fungus. Thus, peat on its own is only good for short term storage of seeds requiring moist cold stratification.

Results of a study by Muller and Bonnet-Masimbert (1984) working on beechnuts (*Fagus sylvatica* L.), found that a pretreatment with polyethylene glycol(PEG), together with chilling at 3°C without any supporting medium was better than stratification in peat, as long as the

moisture contents was maintained at 30 % of seed weight (fresh weight) throughout the experiment. This should also be tried on rowan seed in future experiments.

3.5.7 Behaviour of dormant embryos of seeds stratified at 4°C for various periods of time.

The effects of 7 days and 28 days incubation at 20°C in continuous light of embryos excised from seeds that had been stratified for four periods in polythene bag at 4°C are illustrated in Fig. IV.8 (A, B, C and D) and Fig.IV.9 (A, B, C and D).

A. Cotyledons remain unchanged (white/cream in colour) (Fig IV.8a)

Excised embryos from seeds that had been stratified for 4 weeks had a significantly ($P < 0.1$) higher percentage (36 %, Fig.IV.8a) of embryos unchanged after 7 days of incubation at 20°C. Whereas, embryos after 6, 8 and 10 weeks stratification had fewer unchanged embryos, there was also no significant difference between 6, 8 and 10 weeks stratification. The lowest percentage recorded was 2 %, on the 8 th week of stratification.

Twenty-eight days of incubation, at 20°C resulted in a marked decrease in the percentage of embryos that remained in category A ,for all the four periods of stratification studied (Fig.IV.9a) ,compared to those in this category after only 7 days incubation. There was no difference between stratification periods. However, embryos from seeds that were stratified for four weeks had 12 % at A after the end of the incubation period, while those stratified for 6, 8, and 10 weeks produced only 4, 2 and 4 % respectively.

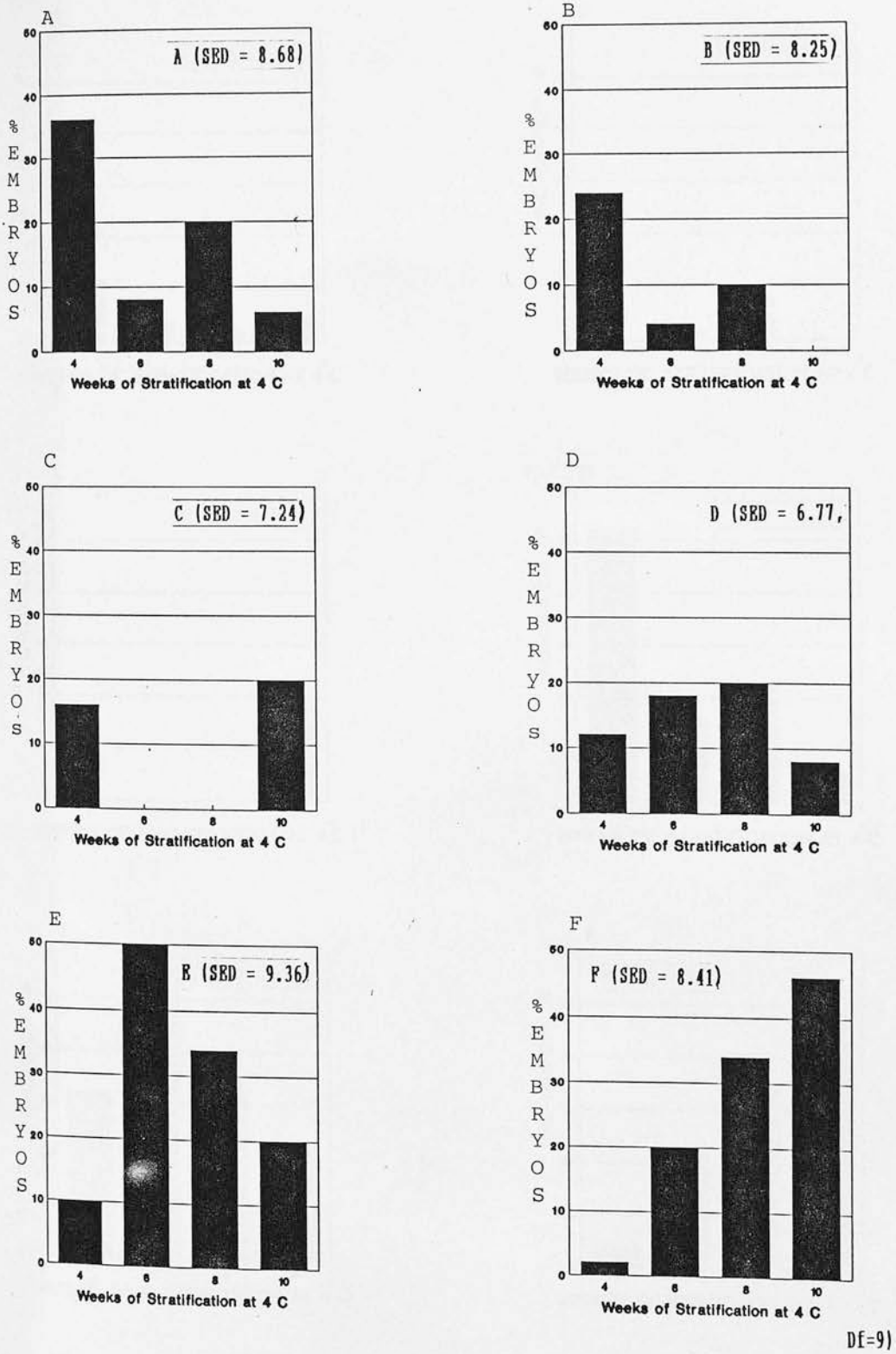


Fig. IV.8 : Percentages of various types of cotyledon greening (A, B, C, D and E) and germination (F), following one week at 20°C and continuous light.

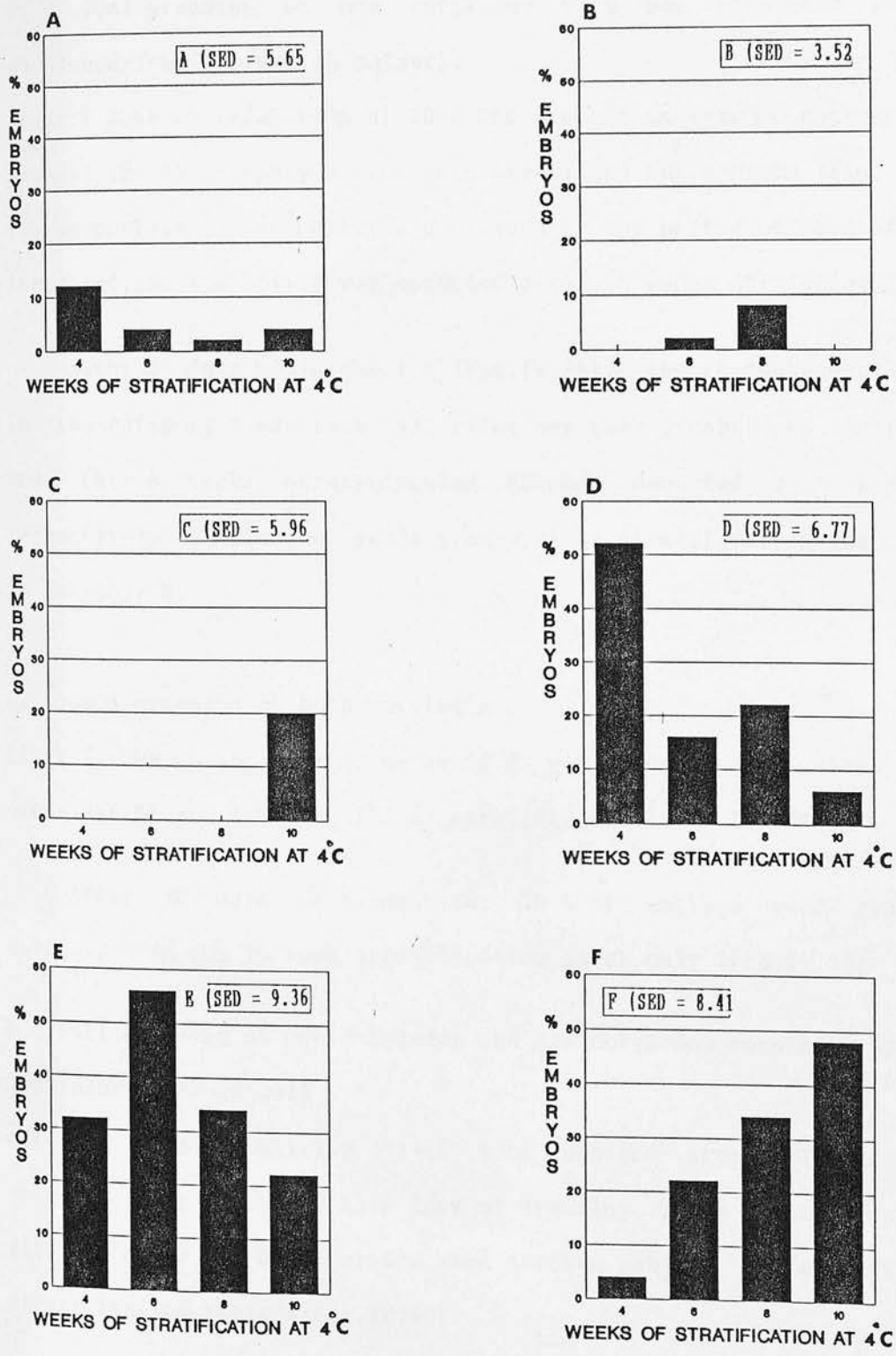


Fig. IV.9 : Percentages of various types of cotyledon greening (A, B, C, D and E) and germination (F), following four weeks at 20°C and continuous light

B. Semi-greening of one cotyledon with one cotyledon remaining unchanged (white/cream in colour).

After 7 days of incubation at 20°C the excised embryos in category B was highest (24 %) in embryos from seed stratified for 4 weeks (Fig. IV.8B). The percentage of such embryos decreased as the period of stratification increased,and the lowest was recorded after 10 weeks stratification(0%).

After 28 days of incubation (Fig.IV.9b), the percentage of embryos in the category B was less than after one week incubation. Only the 6 and the 8 weeks stratification batches recorded 2 % and 8 % respectively, whereas, no seeds from 4 weeks stratification was recorded in category B.

C. Semi-greening of both cotyledon

After 7 days of incubation, category C, was recorded only twice, after 4 weeks (16 %) and 10 weeks (20 %) stratification (Fig.IV.8C).

After 28 days of incubation, 20 % of embryos were placed in category C in the 10 week stratification batch only (Fig.IV.9C).

D. Full-greening of one cotyledon and one cotyledon remaining unchanged (white/cream in colour)

After 7 days incubation, 14.5 % of embryos from all periods of stratification exhibited this form of greening. There was no significant difference ($P < 0.1$) between seed batches subject to the different stratification regimes(Fig.IV.8D).

After 28 days incubation (Fig.IV.9D), category D occurred in a mean of 24 % of seed for all stratification periods with, 52 % of embryos in category D at 4 weeks stratification. Whereas similar percentages were

recorded as for seeds subject to 7 day incubation, for the 6, 8 and 10 weeks stratification periods respectively.

E. Full-greening of both cotyledons

After 7 days incubation at 20°C, 10 % of the four week batch, stratification of seed had complete greening of their cotyledons. Whereas 50 % of the 6 week stratification batch, 34 % of the 8thweek batch and 20 % of 10thweek batch had completely green cotyledons.

After 28 days incubation at 20°C, the 4th and 6thweek batch had 32 % and 56 % of seeds with complete cotyledon greening, respectively. Whereas, the 8 and 10thweek remained about the same level as seed subject to 7 days incubation (Fig.IV.9E).

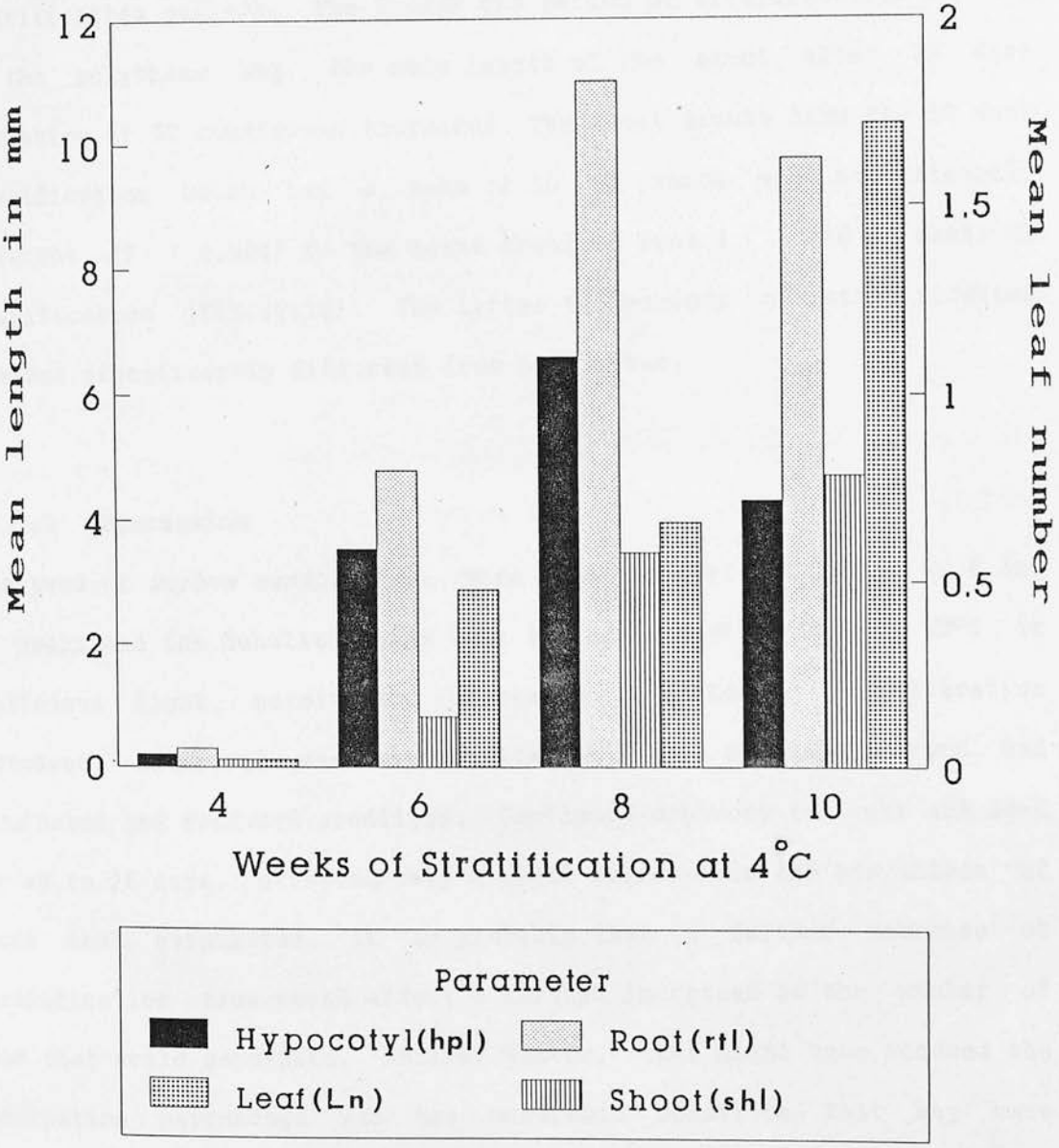
F. Germinated embryos

After the 7 days of incubation, some germination with radicle emergence was observed in all batches (Fig IV.8F). After four weeks stratification, germination was only 2 %, whereas, after 6, 8 and 10 weeks stratification germination increased to 20, 34 and 46 % respectively.

After 28 days of incubation, an almost similar distribution of germination pattern was observed which was on average 2 % higher in the 4, 6 and 10 week batches compared to the germination percentage obtained after 7 days incubation.

3.5.7.1 Growth of seedling hypocotyl, root, shoot and leaf number.

The results of the analysis of variance of the mean hypocotyl length and root length (Fig.IV.10) produced at four periods of stratification were found to be not significant. The mean shoot length recorded ,however,



Sed = 1.382(Hpl),Sed = 2.411(Rtl)
Sed = 0.2443(Ln),Sed = 0.920(Shl
Df = 3

Fig. IV.10 : Mean growth in length of hypocotyls, roots, shoots and mean number of leaf formed by embryos excised from rowan seeds that had been stratified for 4, 6, 8 and 10 weeks at 4°C stratification temperature

were found to be significantly different ($P < 0.001$) between the stratification periods. The longer the period of stratification at 4°C in the polythene bag, the mean length of the shoot after 28 days incubation at GT conditions increased. The shoot growth from the 10 week stratification batch had a mean of 10 mm which was significantly different ($P < 0.001$) to the means obtained from 4, 6 and 8 weeks of stratification (Fig.IV.10). The latter two periods of stratification were not significantly different from each other.

3.5.7.2 Discussion

When seed of *Sorbus aucuparia* L. were stratified at 4°C for 4, 6, 8 and 10 weeks and the dehulled seeds left to incubate at a constant 20°C in continuous light, germination increased as time of stratification increased. After 10 weeks stratification, 46 % of the embryos had germinated and produced seedlings. Continued exposure to light and 20°C for up to 28 days, effected only a small increase in the percentage of seeds that germinated. It is probable that a further increase of stratification time would effect a further increased in the number of seed that would germinate. Another factor, that might have reduced the germination percentage was the anaerobic condition that may have occurred in the polythene bags where the seeds were stratified. However, the tetrazolium and excised-embryo tests performed on the seedlots prior to the experiments gave a viability value of 62 %. and 73 % respectively.

Cotyledon greening was generally greater after incubation for 28 days rather than for only 7 days. However, the cotyledon greening categories reflect the deep embryo dormancy of each seed, which needs a

longer stratification time for dormancy breaking. The first to germinate were the less dormant and this could be a criteria for selecting the best seedlings for propagation. It would have been interesting to investigate the effect of incubating in the dark.

Flemion (1931) also recorded greening of cotyledons that did not germinate after stratification. She showed that in many cases those cotyledons which were in contact with the moist paper enlarged to several time their original size, while the other cotyledons of the same seed not in direct contact with the wet medium were unchanged. Flemion (1931) also found that non-afterripened embryos did not produce seedlings when placed on moist paper, but when put in aerated water, 20 - 40 % of seeds germinated in 2 weeks. Perhaps oxygen is one of the limiting factors that prevents germination in dormant embryos. Bulard (1985) working with apple embryos, whose behaviour may be similar to that of rowan, found that the distal part of the two cotyledons become green first and progressed towards the proximal part. This was also observed for rowan embryos.

The analysis of variance of mean shoot length against period of stratification showed better growth with increasing period of stratification, however, these were mean values, and were therefore dependent on their respective germination percentages. In reality, the growth in shoot length of the after-ripened embryos was no different from each other.

3.6 The effects of Plant Growth Regulators (PGR's) on dormant excised embryos of *Sorbus aucuparia*

3.6.1 Cultivation *in vitro* and the effects of cytokinin and auxins on dormant seeds or embryos (Experiment 10).

(i) Germination of intact seeds

No germination was observed after incubation for one month for any of the treatments.

(ii) Cotyledon greening

Cotyledons of dehulled seeds placed on media with no PGR added showed complete greening in 70 % of cases, the remaining 30 % of seeds possessed only one green cotyledon and the other cotyledon remained unchanged both in size and colour (remained white). Treatment with IAA or BAP resulted in complete greening in all cases, whereas treatment with NAA or IBA resulted in complete greening in 80 % of cases. Seeds placed on GA3 did not develop and senesced within 7 days.

(iii) Embryo development

Treatment with auxins generally resulted in a decline ($P < 0.001$) in hypocotyl extension and shoot growth ($P < 0.001$) for NAA (Fig. IV.11c) and IAA (Fig. IV.11d), N.S. for IBA (Fig. IV.11b) compared to the control (Fig. IV.12a, 12c). Only 12 % of embryos produced both an expanded hypocotyl, root and shoot in NAA (at 0.1 and 1 mg/l), compared to 16 % with IAA (at 1 mg/l) and 8 % with IBA (at 2 mg/l).

Treatment with IAA (5 mg/l) significantly ($P < 0.1$) enhanced root growth (average length 3.4 mm), this was the result of a large increase

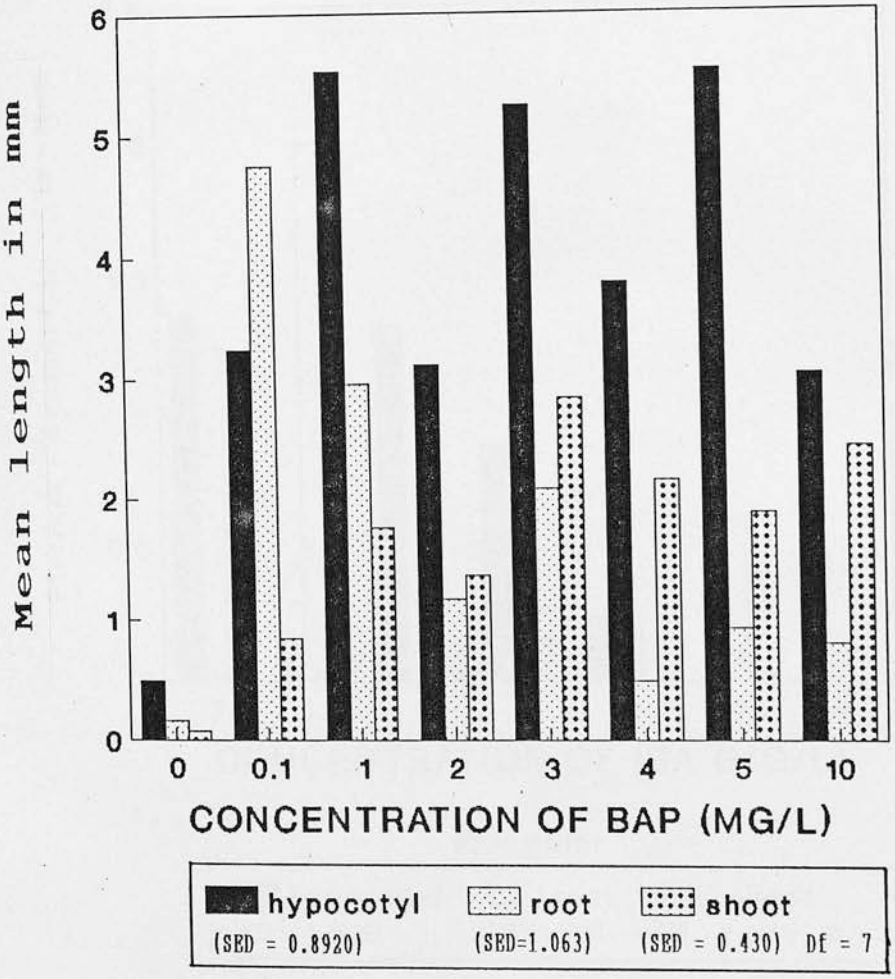


Fig. IV. 11a : Effect of BAP treatments on embryos of *Sorbus aucuparia* after incubation in 20°C with continuous light for one month.

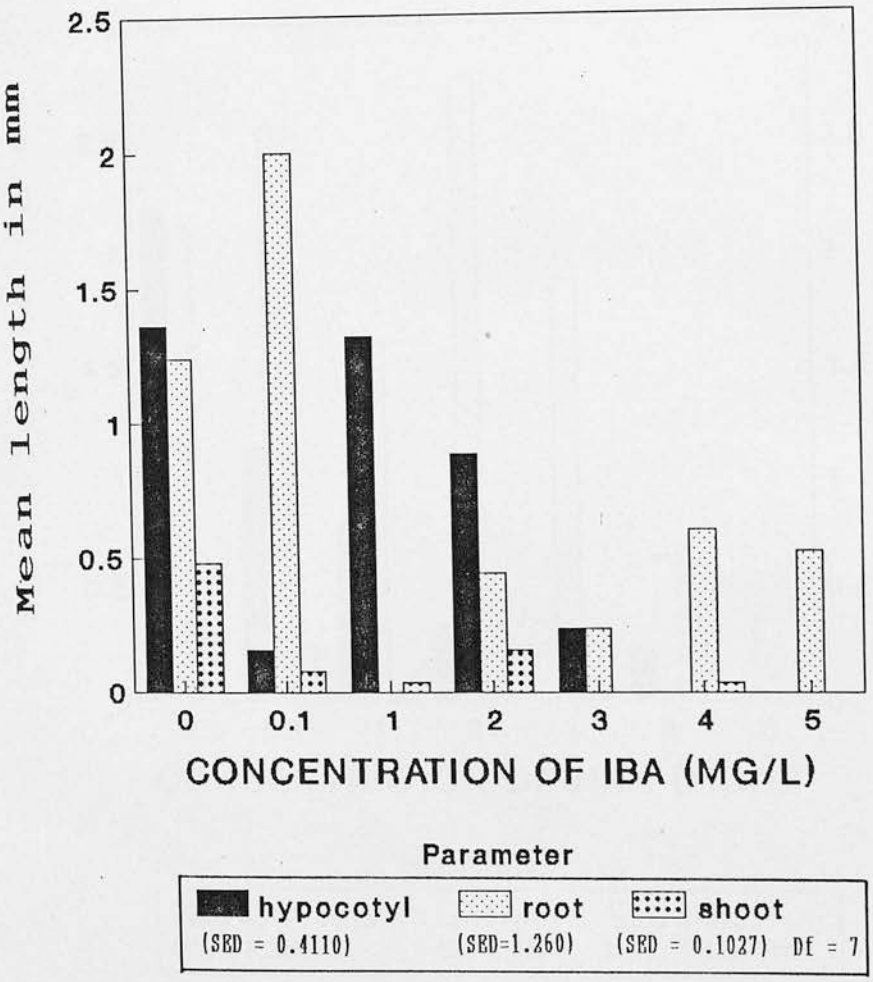


Fig. IV.11b : Effect of IBA treatments on embryos of *Sorbus aucuparia* after incubation in 20°C with continuous light for one month

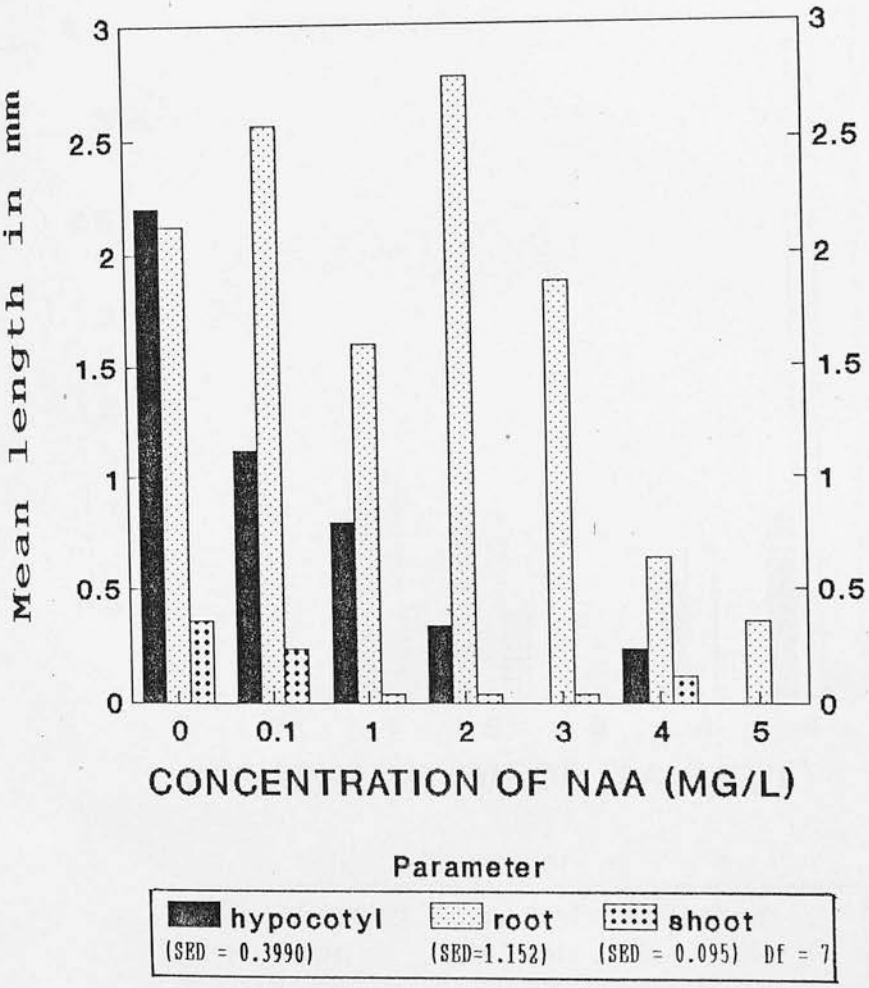


Fig. IV.11c : Effect of NAA treatments on embryos of *Sorbus aucuparia* after incubation in 20°C with continuous light for one month

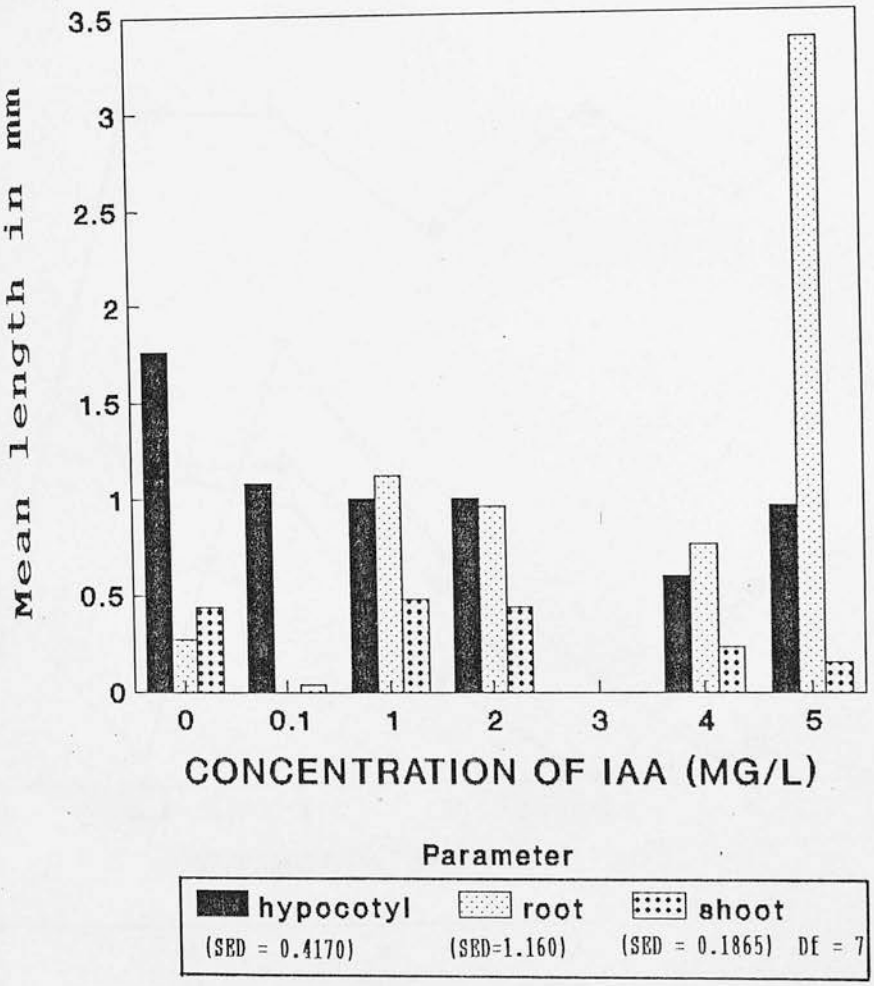
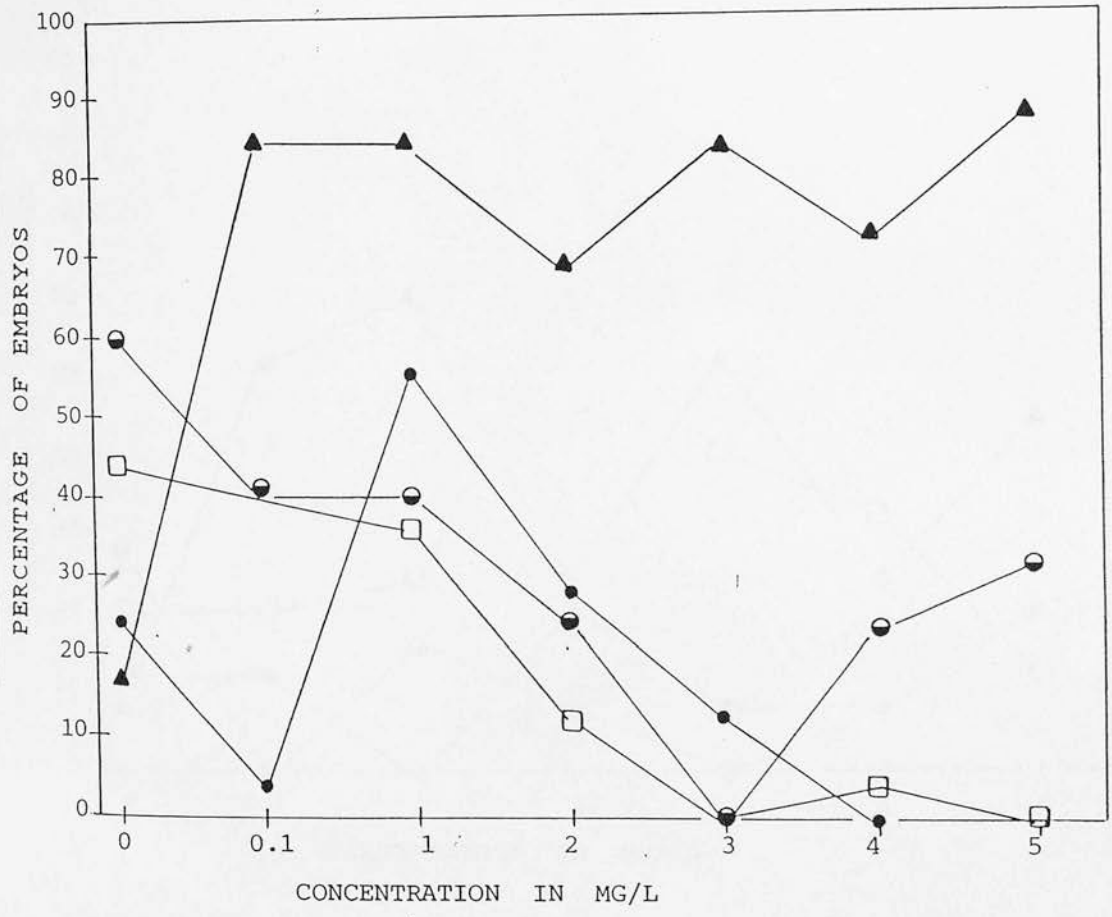


Fig. IV.11d : Effect of IAA treatments on embryos of *Sorbus aucuparia* after incubation in 20°C with continuous light for one month



KEY	▲ BAP	◒ IAA	● IBA	□ NAA
SED = 0.892	0.417	0.920	0.399	
Df = 6	6	6	6	

Fig. IV.12a : Percentage of embryos exhibiting hypocotyl growth in varying concentrations of Plant Growth Regulators (PGRs) in-vitro.

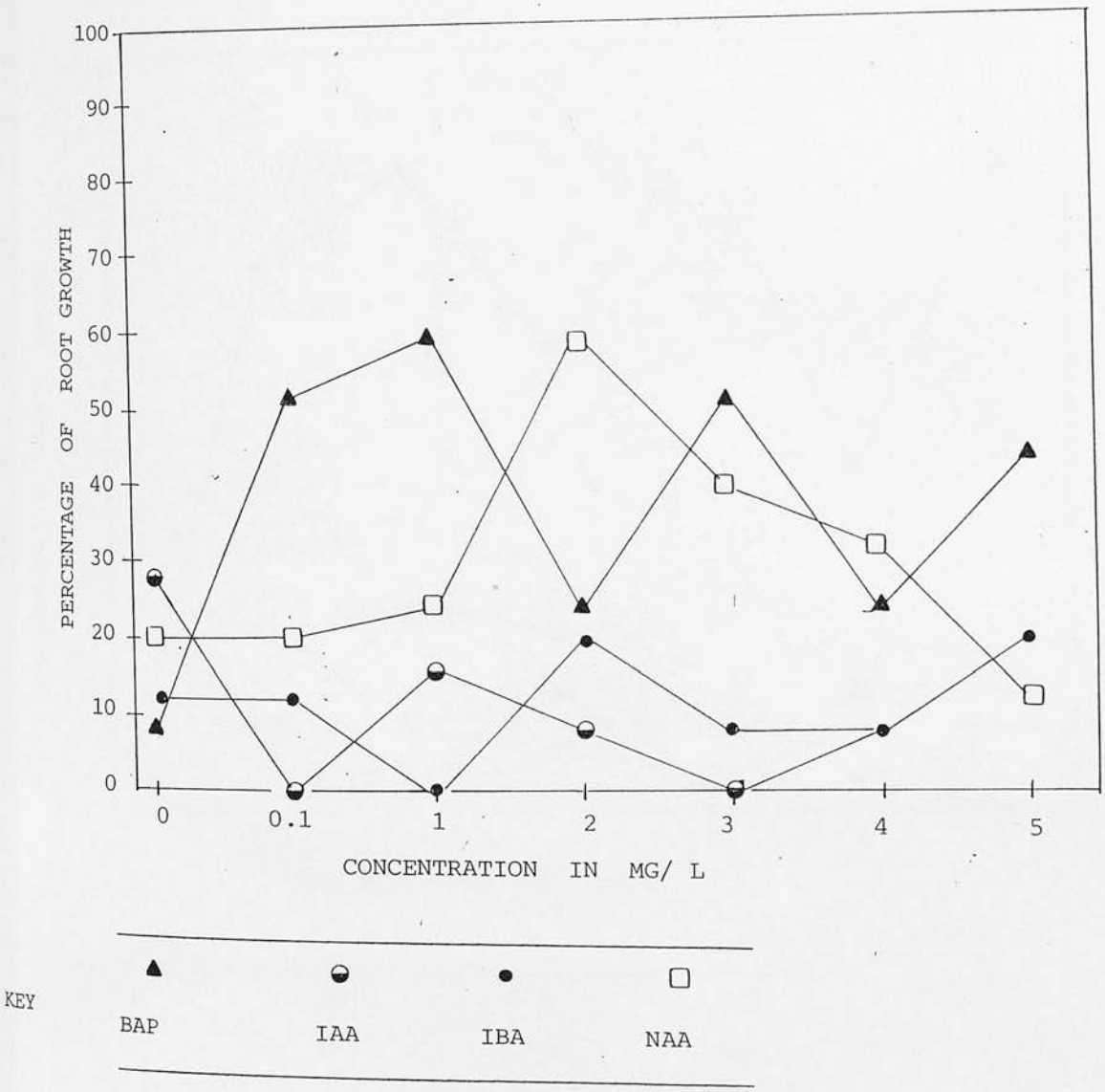


Fig. IV.12b : Percentage of embryos exhibiting root growth in-vitro, subject to differing concentrations of Plant Growth Regulators (PGRs)

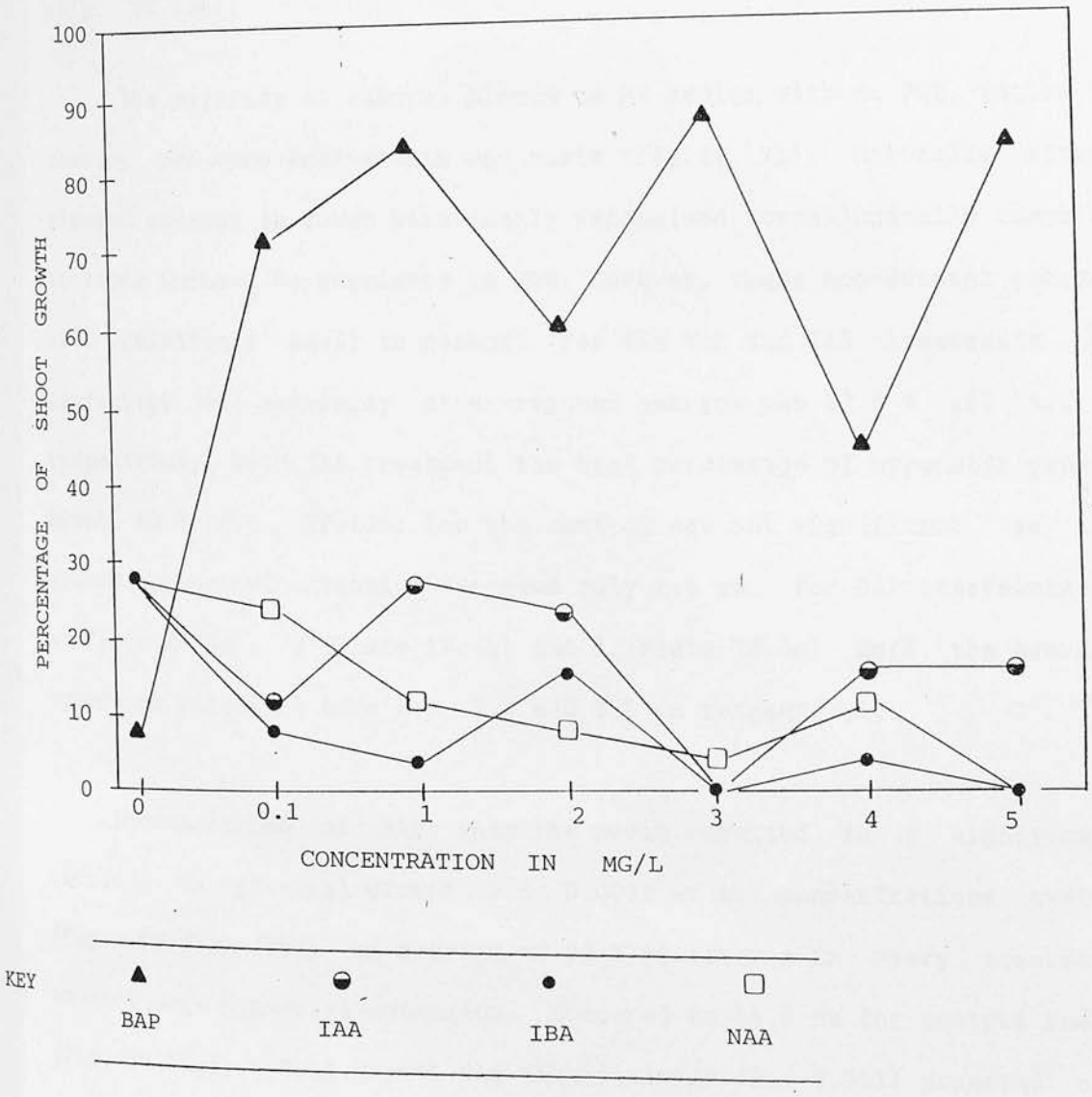
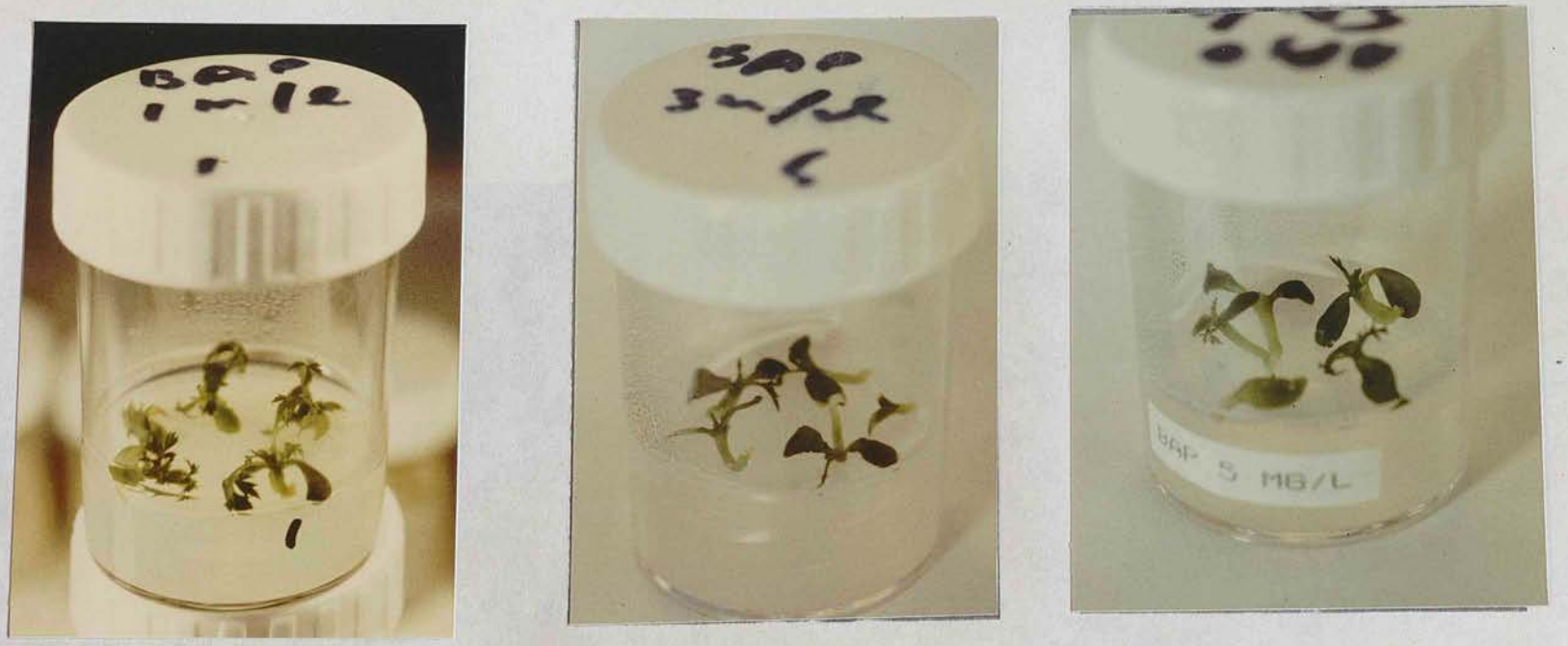


Fig. IV.12c : Percentage of embryos exhibiting shoot growth after incubation in-vitro for one month in varying concentrations of Plant Growth Regulators (PGRs)

in roots in only 20 % of the seeds. Whereas increases in root growth due to NAA (2 mg/l, mean root length 2.8 mm; 3mg/l, mean root length 1.9 mm) were less dramatic but occurred in 60 % and 40 % of embryos respectively (Fig. IV.12b).

The majority of embryos placed on MS medium with no PGR, failed to develop and were dead within one month (Fig.IV.12b). Naturally after-ripened embryos in rowan were easily recognised morphologically compared to those induced to germinate in PGR. However, these non-dormant embryos were relatively small in number. For the NAA and IBA treatments the percentage of naturally after-ripened embryos was 12.5 % and 5.2 % respectively. With IAA treatment the high percentage of hypocotyl growth about 60 % (Fig. IV.12a) for the control was not significant, as the average hypocotyl extension averaged only 1.8 mm. For BAP treatments at 1 (Plate IV.4a), 3 (Plate 1V.4b) and 5 (Plate IV.4c) mg/l, the average hypocotyl extension were 5.5, 5.2 and 5.1 mm respectively.

Incorporation of BAP into the media resulted in a significant increase in hypocotyl growth ($P \leq 0.001$) at all concentrations tested (Fig. IV.11a) with an average of 80 % of embryos in every treatment showing some hypocotyl extension, compared to 16.5 mm for control seeds (Fig. IV.12a). Root growth was significantly ($P < 0.001$) promoted only at BAP concentrations of 0.1 or 1 mg/l (Fig. IV.11a; Plate IV.4a), with between 50 % and 60 % of embryos showing some root growth compared to only 8 % for control (Fig. IV.12b). The number of shoots was significantly increased ($P < 0.001$) by BAP treatment (Fig.IV.11a; Plate IV.4c), on average of 76 % of all embryos subjected to treatment with BAP showing some shoot growth (Fig. IV.12c).



(a) (b) (c)

Plate IV.4 : Incorporation of BAP into MS media promoted hypocotyl, root and shoot extensions in dormant rowan embryos (S. aucuparia L.)
(a) 1 mg/l BAP treatment
(b) 3 mg/l BAP treatment
(c) 5 mg/l BAP treatment, evidenced of multiple-shoots at higher concentration of BAP

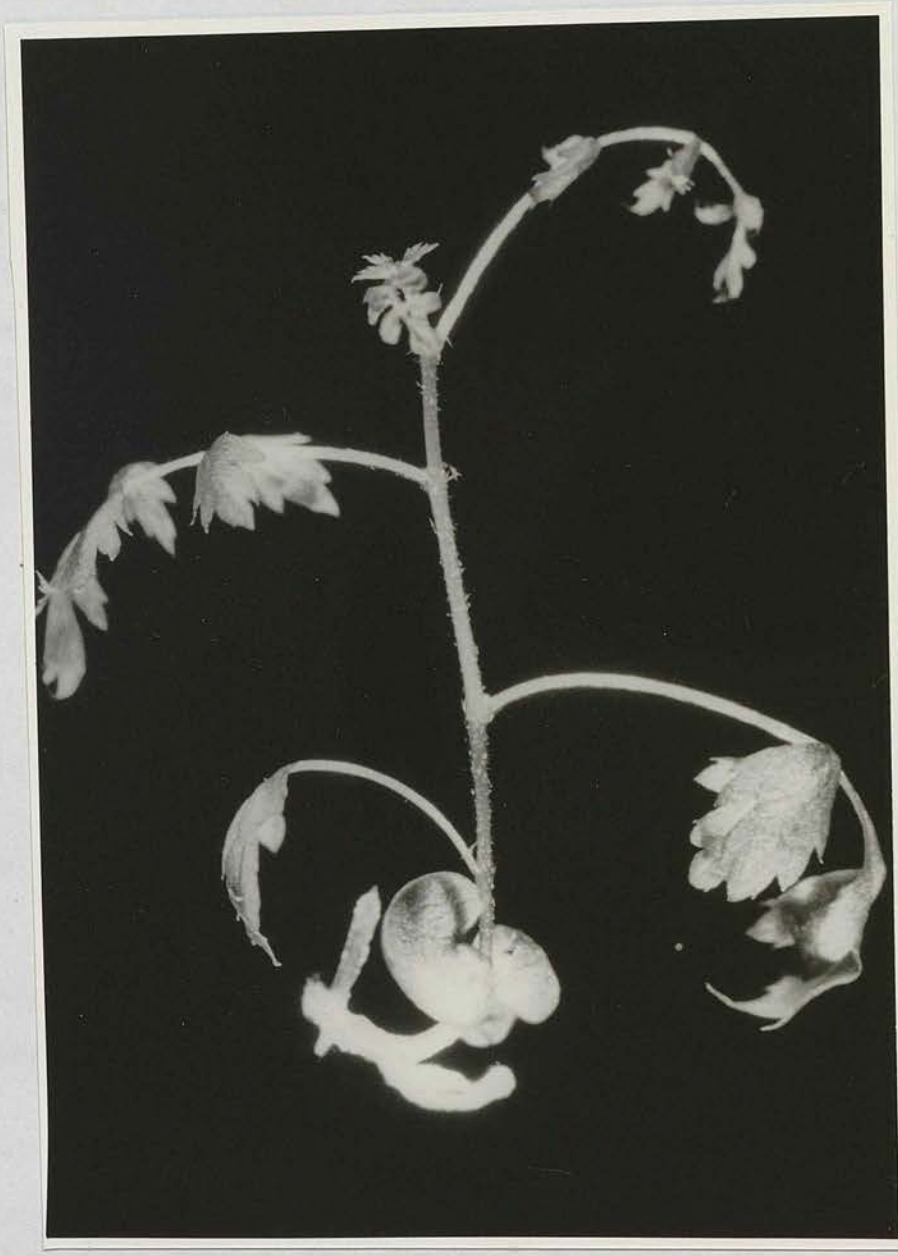


Plate IV.5 : Four months old seedlings of S. aucuparia L. obtained by in-vitro cultivation of dormant embryos incorporating 0.1 mg/l BAP in basic MS medium.

The treatment, after 4 weeks incubation , that produced seedlings most closely resembling those of seedlings after-ripened for 4 months was BAP at 0.1 mg/l (Plate IV.5).

3.6.1.1 Discussion

In *Sorbus aucuparia* L. exogenous application of a low concentration of the cytokinin BAP can closely reproduce the effects of chilling in dehulled seeds, whereas gibberellin (GA3) treatment had no effect on embryo development. This is in contrast to work performed on apple embryos, where there was some relationship between gibberellin concentration and germination, but not with a kinetin treatment (Bulard, 1985).

Auxin treatment was generally ineffective at inducing growth in embryos, although NAA treatment could, promote root growth, as could BAP treatment. It would therefore appear that, at least in rowan, auxin plays little role in breaking dormancy.

It is interesting to note again, as observed in previous experiments that dormancy between seeds differs in intensity. Different seed lots are always heterogenous which greatly complicates the study of dormancy (Come, 1980/1981).

Incubation of whole seeds *in vitro* did not result in germination. Further work is therefore required to elucidate the mechanism of coat-imposed dormancy.

The results do indicate a role for cytokinins in dormancy breaking in rowan. Although more work is required with respect to further cytokinin treatments on intact seeds.

Table IV.8 : The percentage of greening of cotyledons ((i) & (ii)), germination ((iii), (iv) & (v)), embryos with shoot but not root (vi) and unchanged embryos (vii) after 28 days in Germination Test (GT) condition treated with various BAP concentrations

BAP concentration (mg/l) / Percentage (%)	0	0.1	1	2	3	4	5	LSD (Df = 9)
(i) greening of embryos inclusive of those that germinated[1]	66a[2]	64.5a	70a	100b	96b	100b	94.5b	22.836
(ii) embryos fully green + semi-green but ungerminated	88	88	64	0	40	24	40	
(iii) embryos with root + hypocotyl + shoot	0	8	4	80	36	48	0	
(iv) embryos with root + hypocotyl	12	12a 4	12a 0	4a 0	80b 0	44ab 12	60ab 0	0a 46.967
(v) embryos with hypocotyl + shoot	0	0	8	0	4	0	0	
(vi) embryos with shoot but not root	0a	0a	24ab	20ab	12a	16ab	60ab	47.557
(Vii) embryos that remained unchanged	0	0	8	0	4	0	0	

1. Mean percentage of greening of embryos
2. Percentage in the same row followed by the same letter are not significant at 0.05 level of significance

3.6.2 The development of embryo growth on paper substrate wetted with BAP at various concentrations (Experiment 11)

The effects of incubating dehulled seeds on paper substrates wetted with the above cytokinin at GT conditions for 28 days produced various degrees of greening of cotyledons and germination. Some remained fresh fully green or semi-green and ungerminated. Some that germinated, either produced both shoot and root or only shoot growth but not root. A few remained creamy white totally unchanged though slightly enlarged.

(A) Greening of cotyledons

Treatment of embryos with various concentration of BAP solutions have shown a range of greening of the cotyledons, some with 100% greening of both cotyledons whereas others only had semi-green cotyledons. Greening began from the distal end of the cotyledons. Semi-green embryos usually had no greening at the proximal end near the radicle.

The analysis of cotyledon greening were categorised into two groups. Firstly, all embryos that had cotyledon greening after the incubation period inclusive of those that later germinated or produced only shoot growth were estimated for its percentage of greening of both cotyledon. The mean percentage greening of all 25 embryos per treatment were calculated (Table IV.8 (i)). Secondly, embryos that germinated or produced shoot were excluded and the mean of the percentage greening of either fully green or semi-green embryos but remained ungerminated after incubation were again recalculated (Table IV.8 (ii)).

In the first group, the percentages of greening of all embryos per treatment were found to be significantly increased ($P < 0.05$) with

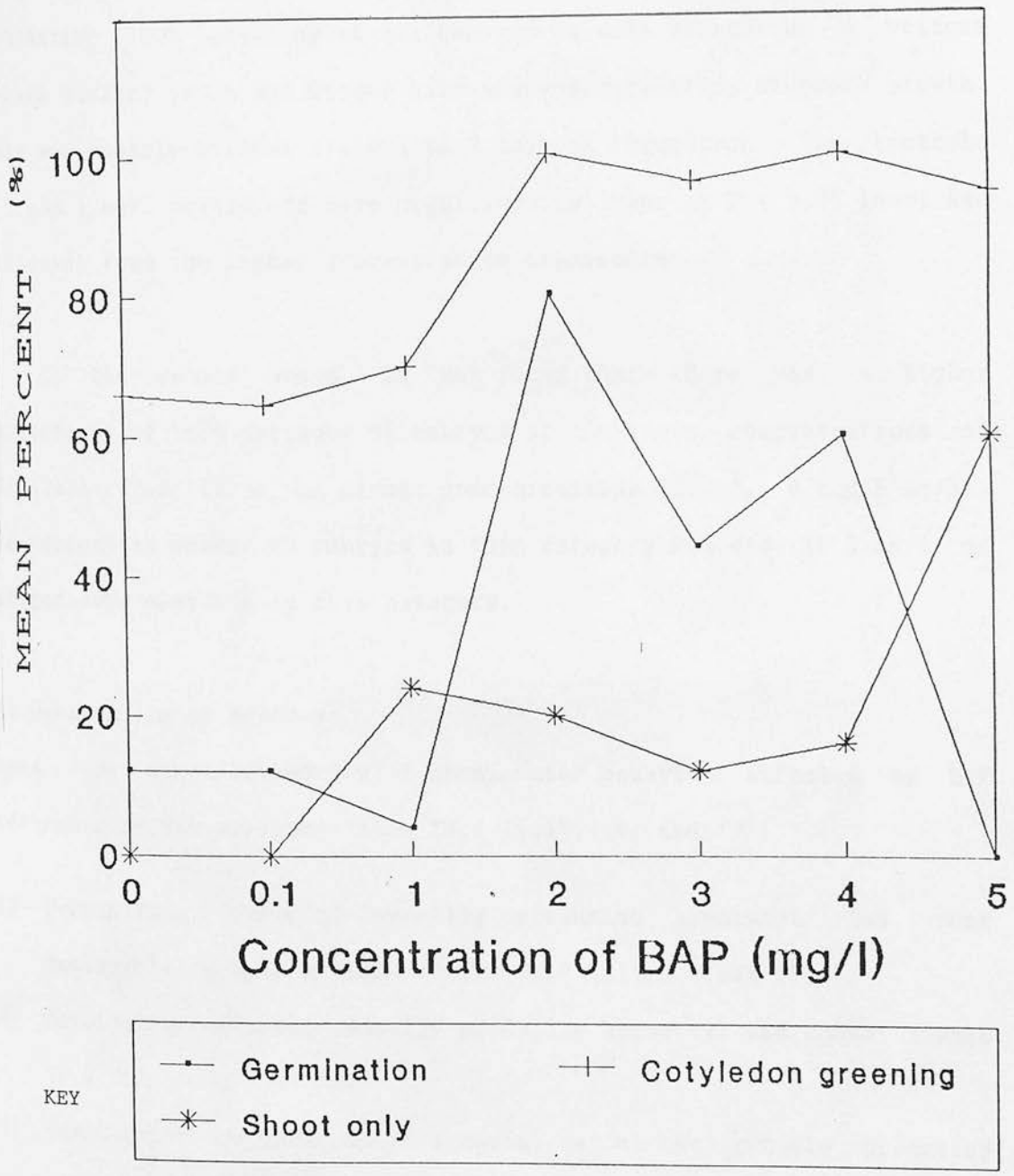


Fig. IV.13 : Effect of BAP treatments on embryos of rowan on percentage of germination, greening of cotyledons and embryos that produced shoot but not root (LSD & Df values refer to Table IV.8).

increasing BAP concentration (Table IV.8 (i), Fig.IV.13). However, 2 mg/l treatment produced the best percentage of embryo greening exhibiting 100% greening of all embryos as well as showing a uniform darker healthy green and bigger size embryos reflecting vigorous growth. This was clearly evident after 5 to 7 days of incubation. The control, 0.1 and 1 mg/l treatments were significantly lower at $P < 0.05$ level and different from the higher concentration treatments.

In the second group, it was found that there was a higher percentage of this category of embryos at the lower concentrations of BAP (Table IV.8 (ii)). At higher concentrations (2, 3, 4 and 5 mg/l), the percentage number of embryos in this category reduced. At 2 mg/l no embryos were observed in this category.

(B) Germination of embryos

There were three categories of germination behaviour effected by BAP treatments on the embryos (Table IV.8 (iii),(iv) and (v)).

(1) Germination occurred normally producing hypocotyl and root extensions as well a shoot (Table IV.8 (iii), Plate IV.6).

(2) Germination occurred normally producing hypocotyl and roots (Table IV.8 (iv)) and

(3) Germination occurred in an abnormal way at the radicle producing only hypocotyl extension but no root but with a normal shoot (Table IV.8 (vi), Plate IV.7).

In the entire experiment, only a small percentage of the embryos germinated fell into category (2) and (3)(Table IV.8 (v). Whereas normal germination, category (1) predominated in all treatments tested, with

Plate IV.6 : Germination of dormant embryos by exogenous application of BAP wetted on paper substrate.

Plate IV.7 : Embryos germinated after BAP treatment that produced shoot and hypocotyl but not root (top left)

the 2 mg/l treatment effecting the highest percentage germination (80 %) (Table IV.8 (iii), producing healthy seedlings (Plate IV.6). The control and the lower level of concentration treatments (0.1 and 1 mg/l) had significantly lower percentages of germination. And at 5 mg/l , no germination was recorded following the definition of germination adopted in this study.

Besides effecting a normal germination, exogenous application of BAP on excised embryos stimulated the embryos to produce shoots without any roots being formed (Table IV.8 (v)). This were observed at 1 mg/l concentration and above. Concentration of 5 mg/l of BAP effected the highest percentage (60 %) of embryos producing shoot but no root.

(C) Embryo development in mean length of root, hypocotyl and shoot and leaf number.

Overall, treatment with 2 mg/l of BAP resulted in the best growth in all the parameters recorded (Fig. IV.14, Table IV.9). Its mean root length (6.16 mm) was the longest recorded followed by the 3mg/l treatment (5.44 mm) which were not significantly different from each other ($P < 0.05$). Similarly, the 2 mg/l treatment produced seedlings with the longest mean hypocotyl length (6.60 mm) and it was significantly different ($P < 0.05$) as with all the other treatments. As referred to earlier, the 5 mg/l BAP treatment did not promote root or hypocotyl growth. The mean shoot length was longest (2.04 mm) in a media containing 2 mg/l BAP, however, this was not significantly different ($P < 0.05$) to the values obtained with media containing 3, 4 and 5 mg/l BAP respectively (Table IV.9).

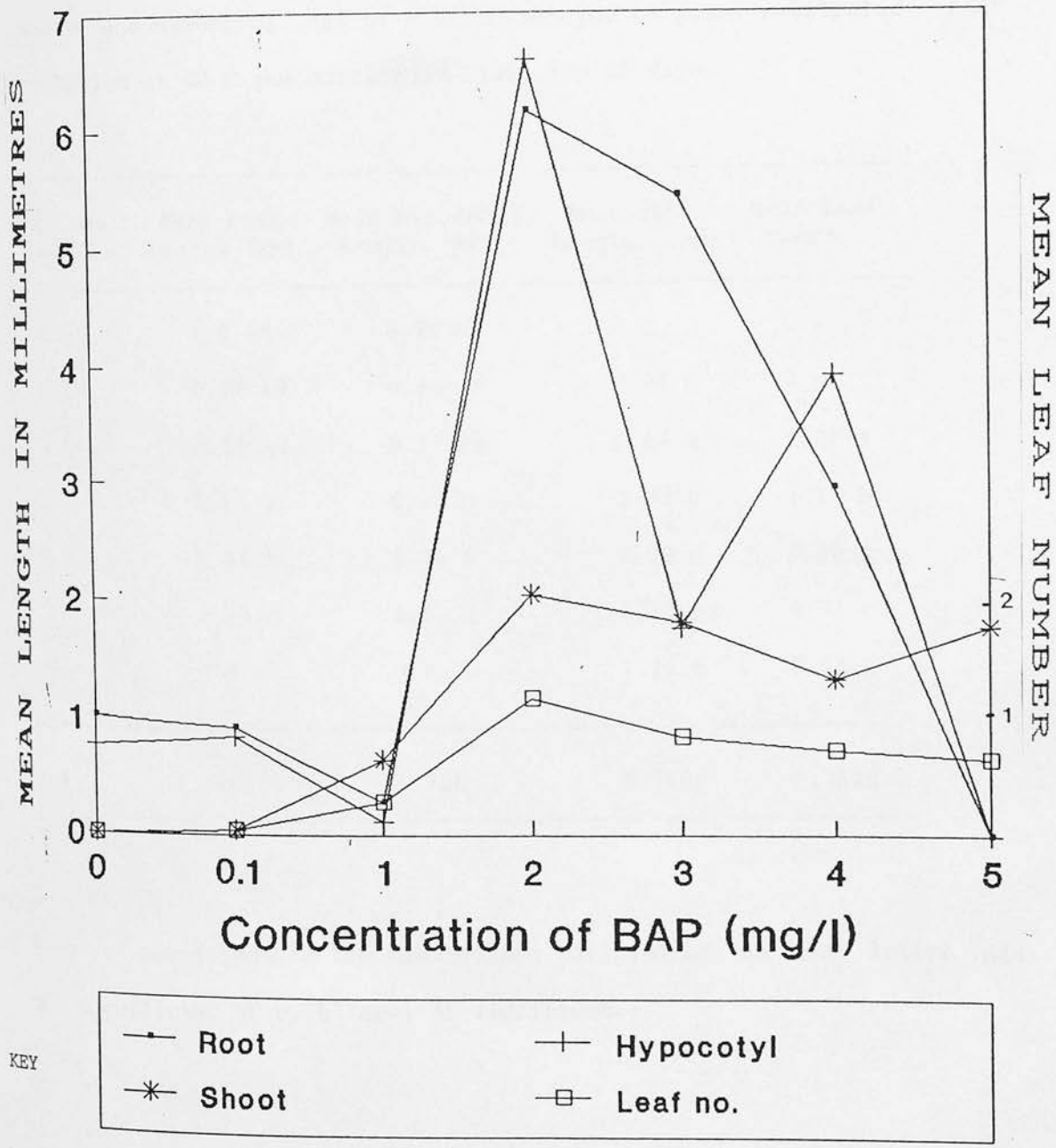


Fig. IV.14: Effect of BAP treatments on excised embryos of rowan on growth of root, shoot, hypocotyl and number of leaf formed after 28 days in Germination Test (GT) conditions. (LSD and Df values refer to Table IV.8)

Table IV.9 : Effects of BAP treatments on growth of roots, hypocotyl, shoots and number of leaf of excised embryos on paper substrates after incubation at 20 C and continuous light for 28 days.

BAP Conc. (mg/l)	Mean Root Length (mm)	Mean Hypocotyl Length (mm)	Mean Shoot Length (mm)	Mean Leaf Number
0	1.0 ad	0.76 ab	0 a	0 a
0.1	0.92 ad	0.84 ab	0.04 a	0 a
1	0.28 ad	0.12 ab	0.64 ab	0.28 a
2	6.16 b	6.60 c	2.04 c	1.16 b
3	5.44 bc	1.76 a	1.80 c	0.84 c
4	2.96 c	3.92 d	1.32 bc	0.72 c
5	0 d	0 b	1.76 c	0.64 c
LSD	2.7461	1.725	0.7606	0.2842

Df = 168

Mean or percentage in the same column followed by the same letter are not significant at 0.05 level of significance.

The number of leaves formed consistently showed that the 2 mg/l BAP treatment produced the most leaves (mean number = 1.16) (Table IV.9).

3.6.2.1 Discussion

The application of exogenous BAP to dormant embryos of *Sorbus aucuparia* was found to break its dormancy as seen in earlier experiments (Section IV.3.6.1). However, a higher concentration of BAP (2 mg/l) was optimal in the range of concentrations tested to remove the deep embryonal dormancy that they possessed when compared to the *in-vitro* method which was optimal at 0.1 mg/l. The contrasting response to 5 mg/l BAP between the two methods of applications is not understood. Perhaps, the continuous wet environment of a petri-dish allowed a faster rate of intake of BAP into the embryo and inhibited growth. In contrast, *in vitro*, water uptake on the agar media was perhaps more controlled, and together with the extra nutrients perhaps slowed the intake of BAP into the embryo and therefore may have reduced the impact of the high dosage.

Other studies have also indicated the importance of CK in breaking the dormancy of seeds, especially in *Acer saccharum* (Webb & Dumbroff, 1969), *A. pseudoplatanus* (Pinfield & Stobart, 1972a; 1972b) and, *Pyrus malus* (Kopecky *et al* , 1975) as discussed earlier (section I.2.2.1.4).

The behaviour of cotyledon greening of *S. aucuparia* described by Flemion (1931) was manifested in the control experiment of my study, whereby excised dormant embryos turned green in the presence of light .

In the control experiment, however, 12 % of the seeds had after-ripened naturally. These after-ripened embryos became green rapidly and germination followed after a week of incubation. In contrast, in the dormant embryos, the greening of cotyledons was much slower and proceeded at various rates. This suggests that there were different depth of dormancy in a seedlot. The behaviour of cotyledon greening was earlier used in apple (*P. malus)* by Bulard (1985) to evaluate the depth of the apple embryo dormancy and this could be similarly applicable for *S. aucuparia*. The greening of cotyledons was enhanced by minute concentrations of BAP (0.1 and 1 mg/l) on paper but at higher concentrations (2, 3, 4 and 5 mg/l) the percentage of cotyledon greening (Table IV.8) approached 95 - 100 %.

All cotyledons greened with 2 mg/l BAP and also, the highest germination (80 %) was also obtained, this was comparable to those obtained by the prechilling treatment (Section IV.3.1 - IV.3.2). This suggests that a similar CK may play a role in the breaking of dormancy in this species . Much research has indicated that the regulation of both dormancy and dormancy breaking is through a balance between endogenous hormonal promoters and inhibitors (Amen, 1968; Pinfield *et.al.* ,1975; El Antably, 1976).

The formation of shoots but no root (Fig IV.14) as a result of exogenous BAP treatments, which tended to be more emphasised as BAP increased suggests that there was a varying sensitivity of the target tissues or organs to the hormone. Perhaps it could be explained that the sensitivity varied according to the depth of dormancy. The 20 % of embryos (Table IV.8 (vi)) producing only shoots with 2 mg/l BAP,

manifesting the effect of a high dose of BAP. It is suspected that it had a lower depth of dormancy compared to the majority of embryos (80% , Table IV.8(iv)). It follows that a lower concentration than the 2 mg/l BAP, was probably sufficient for normal germination.

The mean growth in the length of hypocotyl, root and shoot and the number of leaves obtained per treatment were used as diagnostic features to select the most successful concentration for dormancy breaking. Results showed that after a certain optimal concentration of BAP (2 mg/l), hypocotyl and root extension and/or formation were suppressed (Fig. IV.14), Table IV.9). However, for shoot extension and numbers of leaves formed, the effect were not conclusive, due to the short duration (28 days) in GT conditions on which the data were based. Perhaps continuing their growth further may possibly warrant a better assessment, but this was not done.

3.7 Infusion of plant growth regulators into the seed.

3.7.1 Organic infusion of cytokinin(BAP) on intact seed (Experiment 12)

None of the seeds after treatment germinated.

3.7.1.1 Discussion

The discovery that excised embryos of dormant rowan seeds germinated after treatment with 2 mg/l BAP, led to attempting to infuse the PGR without scarifying and nicking the seed. However, the experiment was not successful. Persson (1988) employed a similar procedure on 47 species. Among the woody species he studied seeds of *Chaenomeles japonica, Malus silvestris, Rosa dumalis, R. canina, Euonymus europaeus* and *Sorbus*

aucuparia were employed. All of these species did not germinate after infusion with germination promoters such as Ethrel, gibberellic acid (GA_3) and kinetin at concentration ranging between 1 um and 10 um. All of these species are known to require long periods of cold stratification.

Probable reasons for this failure to respond to organic infusion are:

(i) seeds may be susceptible to solvent damage as reported earlier by Lewis, Papavizas and O'Neill (1979) on many species.

(ii) Testa and the leathery pericarp of *Sorbus aucuparia* remains impermeable to organic infusion of the cytokinin.

(iii) The PGR may be infused, but the sudden change in the hormonal balance cannot break dormancy by itself.

(iv) The PGR may be infused and the seed may be stimulated to afterripen but the mechanical restriction of the seedcoats may impede further growth.

Attempts by other methods to infuse PGR such as BAP into the seed of *Sorbus aucuparia* should be continued. Other methods such as dissolving the seedcoat by treating with acetone and ethanol, as reported in Anderson *et al* (1953) and Oelke and Albretcht (1980) could be tried.

3.7.2 Investigation to determine whether BAP solution would penetrate the mesocarp and effect ripening in *Sorbus aucuparia* seeds (Experiment 13)

None of the embryos with the mesocarp intact germinated. Whereas the excised embryos responded well to treatment. After 7 days of incubation all embryos exhibited complete cotyledon greening and were healthy. After the end of the experiment 100 % the embryos had after-ripened and germinated into normal seedlings (Table IV.10). Although the seedlings formed were morphologically stout at the hypocotyl and had shorter roots, otherwise they were very healthy with fresh dark green shoots and leaves.

Table IV.10 Percentage of germinations of *Sorbus aucuparia* seeds in 2 mg/l BAP after one month in 20°C with continuous light.

Treatments	% Germination
Testa + mesocarp intact	0
Mesocarp intact	0
Mesocarp nicked	72 ± 28.37
Excised embryos	100 ± 23.481

1 Sample Standard Deviation, n = 5

3.7.2.1 Discussion

The results show that the mesocarp impedes the germination of *Sorbus aucuparia* and embryos responded to the exogenous application of 2 mg/l BAP solution wetted into a filter paper substrate. Flemion (1931) attempted to infuse various chemicals such as potassium cyanide, urea , saponin, vanadium compounds, triethanolamine, organic and inorganic acids, alcohol, aldehydes, glycerine , hydrogen peroxide, adrenalin and cyanate, but all were unsuccessful. Most of these chemicals tested were unable to penetrate into the seed. However, some evidence of embryo injury to certain chemicals was reported which suggested that the seedcoats was permeable at least to certain chemicals but no mention of which chemicals was made.

3.8 Mechanical restriction of embryo growth by seed coverings (Experiment 14)

No germination occurred in the intact seeds but 14 % germination occurred in inner coat intact embryos after four weeks of incubation. Embryos with inner coat nicked have 28 % of the samples recorded cotyledon greening and 2 % of them afterripened producing 4 mm of radicle after 7 days of incubation. Whereas 68 % remained fresh and slightly enlarged (Table IV.11).

After two weeks at incubating temperature the nicked samples began to after-ripen producing radicles and greening of cotyledons. After four weeks, 50 % of them germinated and 48 % remained fresh and unchanged and 2 % showed one cotyledon greening. The mean hypocotyl length and root

Table IV.11 : Percentage of nicked embryos responded to exogenous application of 2 mg/l BAP solution after incubation at 7 and 28 days.

Incubation (Days)	remained unchanged	Cotyledon greening	Percentage Germination
7	68	28	2
28	48	2	50

3.8.1 Discussion

This investigation generally manifested the restrictive and impermeable nature of the testa and the mesocarp to the entry of PGR into the embryos. The significantly higher germination rate of 50 % in the nicked samples of mesocarp intact embryos indicated that 2 mg/l BAP solution had entered and promoted after-ripenning. Micro-operating on very small seeds like *Sorbus aucuparia*, nicking the seed uniformly poses a problem besides taking care not to cause any injury to the embryo. The high percentage of nicked samples remained unchanged at the end of the experiment perhaps attributed to mainly to the cut being too small. If that was true, then the leathery mesocarp could be repressing the after-ripening process promoted by the application of BAP. By the same argument, the nicked samples that after-ripened and continue germinating producing seedlings perhaps could be attributed to the larger cut made to the mesocarp which reduced its restrictive strength to oppose any growth made by the embryos.

3.9 Detection of cytokinin in the embryo, testa and mesocarp at different periods of cold stratification.

3.9.1 Detection of cytokinin in the embryo, testa and mesocarp at different periods of cold stratification (Experiment 15)

There were altogether 4 embryo, testa and mesocarp extracts each, obtained from seeds that has been stratified for 4, 6, 8 and 10 weeks. Unfortunately, the extracts of testa and mesocarps for the ten week stratification period were accidently lost. Therefore, only three extracts each for the testa and mesocarp were used for spotting on chromatographic plates, representing 4, 6 and 8 weeks of stratification sequence.

Two solvent systems were used to run the extracts on a one dimensional chromatogram and the results are shown in Fig.IV.15 and 16 and the cytokinins distribution is summarized in Table IV.12.

In *system 1*, the length of the run was 14.5 cm and time taken to reach it was exactly four hours.

Generally, for each sample tested, a heavily laden spot which was black in colour when sprayed with Wood's reagent, formed just above the origin. These spots were quite large which turned dark reddish brown when left in the open. When analysed with HPLC and comparing to a standard BAP run (Fig.IV.17) it did not produce any peak (Fig.IV.18) at 254 nm wavelength.

Similarly, it was observed that there was a fast moving compound carried to the top of the chromatograms. It occurred more strongly in the embryos at 4 weeks stratification. They were detected both under UV

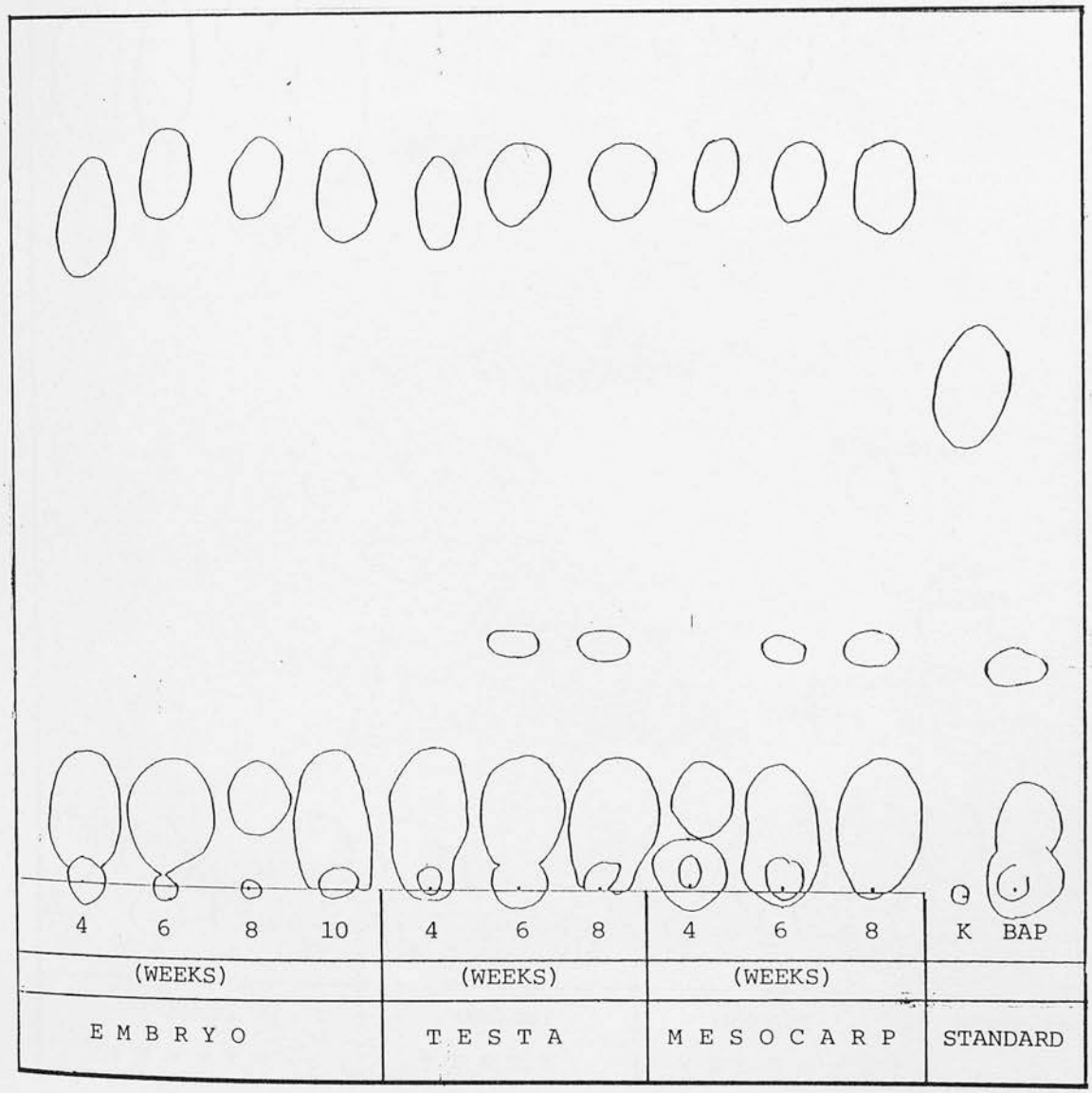


Fig. IV.15 : **Diagram showing the typical distribution on one dimensional 60F254 (solvent A) of cytokinin compounds detected by UV light or Wood Reagent.**

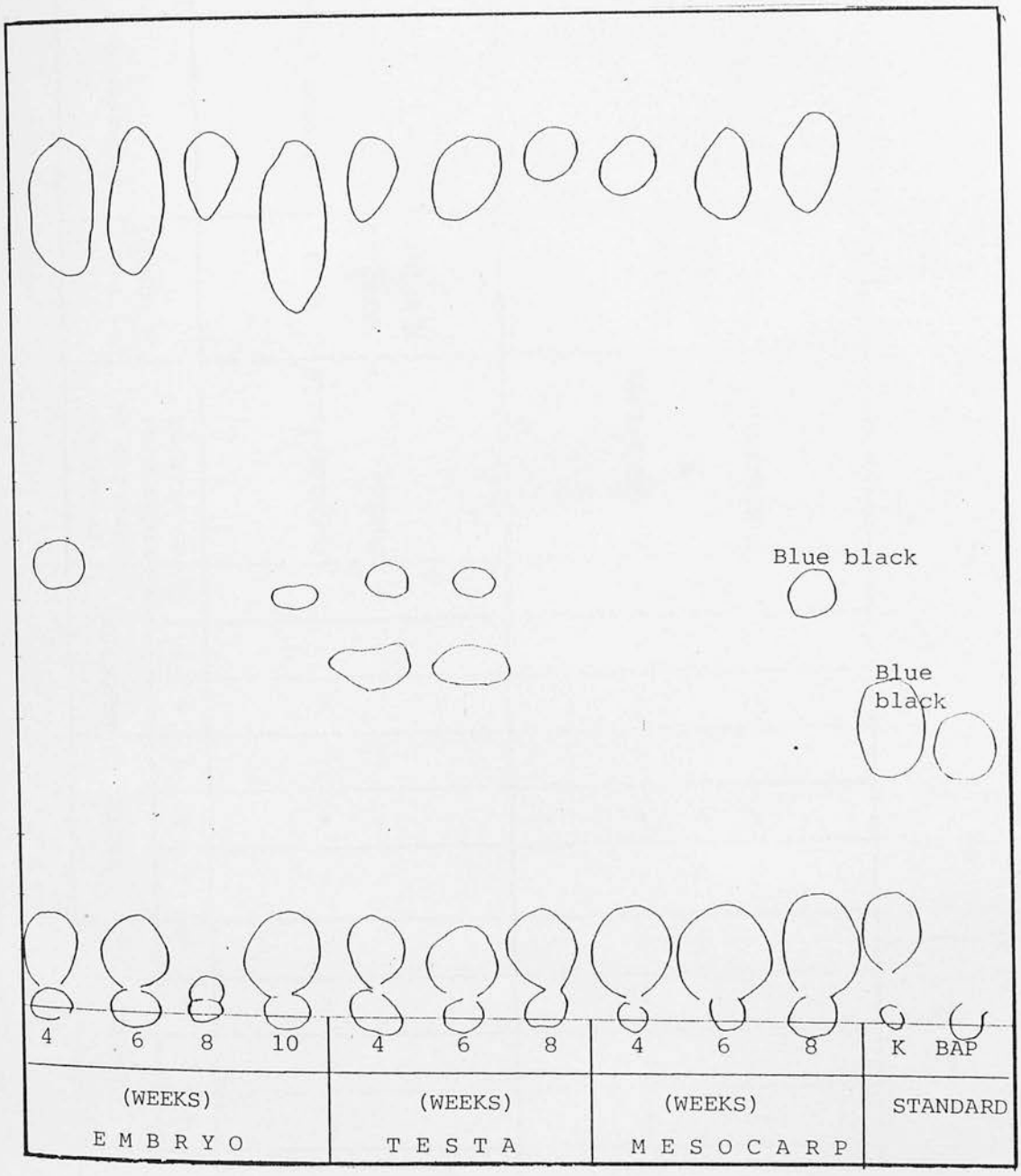


Fig. IV.16 : **Diagram showing the typical distribution on one dimensional $60F_{254}$ (solvent B) of cytokinin compounds detected by UV light or Wood Reagent.**

Table IV.12 : The distribution of endogenous cytokinins extracted from various seed parts from seeds stratified for various stratification periods at 4°C.

SEED PARTS	EMBRYO				TESTA			MESOCARP			Probable identity of endogenous cytokinins	Rf for Standard	References
4°C stratification (wk)	4	6	8	10	4	6	8	4	6	8			
System 1													
Rf = 0.83	++	+	+	+	+	+	+	+	+	+	Dihydrozeatin	0.83	Nitsch (1968)
Rf = 0.25						+	+		+	+	Purine	BAP=0.23 K = 0.53	
System 2													
Rf = 0.82	++	+	+	+	+	+	+	+	+	+	Dihydrozeatin	BAP=0.26 K = 0.28	
Rf = 0.44	+			+	+	+				+	?		
Rf = 0.34					+	+					cis-zeatin	0.32	Leonard et.al. (1971)

at 254 nm wavelength (dark grey) and when sprayed with Wood's Reagent (bluish purple). It had an Rf of 0.83. Another possible cytokinin located at about the same level as the BAP marker (Fig IV.15) was present (Table IV.12), in the *testa* and the *mesocarp* extracts after both *6* and *8 weeks* of stratification. These weak spots located only through UV light which emits purplish-grey colour.

In *system 2* , the length of the run was 17 cm and it took 4.5 h to reach the solvent front. By using chloroform:methanol (9 : 1) as the solvent (Fig.IV.16) two other cytokinins have been detected (Table IV.12) under UV and they were of weak intensity, which were not separated in the first system. It occurred about half-way the length of the chromatogram (Rf = 0.44) and found in five of the samples (see Table IV.12), namely, at embryo (4 and 10 week), testa (4 and 6) and mesocarp (8 week). Below the above spots, another spot (Rf = 0.34) coloured darker pink under UV light ,located at the testa extract position 4 and 6 weeks stratification. A similar cytokinin as found in *system 1* ,was also observed in system 2 . Essentially, their distribution were similar, at the top of the chromatogram and having an Rf of 0.82 (Fig.IV.16) and Table IV.12.

Fig.IV.17 : Cytokinin (BAP) standard run on HPLC

Fig.IV.18 No peak was obtained for the black spot found near the origin of each samples.

3.9.2 Discussion

This preliminary survey on the occurrence of endogenous cytokinin in *S. aucuparia* seeds, at various periods of 4°C stratification, perhaps provide some evidence of their presence. There may be four cytokinins recognised from both solvent systems. No report has been found up to date on cytokinin in seed of *S. aucuparia*.

In this investigation, no attempt was made to actually identify them by separately extracting, fractionating and re-chromatographing, as it was felt that this was beyond the scope of my study. Such separations are normally followed by bioassay of segments of the chromatogram to identify active materials (Audus, 1972). Therefore, in this investigation, the identification of each cytokinin, if at all possible, were merely speculative and based on comparative Rf values of reports from literature.

The fastest moving cytokinins with an Rf values of 0.83 or 0.82 which occurred in all the seed parts investigated, matches well with those described by Nitsch (1968) and its identity could be dihydrozeatin. Dihydrozeatin is a close relative of zeatin (0.80, Nitsch, 1968) and has been isolated from immature *Lupinus luteus* seeds (Koshimuzu *et al*, 1967) and was also the metabolite identified by TLC on solvent A in sweet corn kernels or caryopses (Summons *et al*, 1980). In this study , dihydrozeatin was found in all parts of the seeds regardless of the weeks of stratification that they were exposed to. This suggest that its amount was quite significant and it was evident that chilling did not influence its distribution in the seed parts.

However, at the embryos, there were deeper staining with $AgNo_3$, compared to those of testa and mesocarp, particularly, the spot from the 4 weeks batch. It appears that, dihydrozeatin decreases in its concentration as chilling progresses towards the eight weeks, as seen from the lighter staining with $AgNo_3$ on the chromatogram. This may imply that it may be activated by protracted chilling and utilised in the embryos. Dihydrozeatin has been known to be abundant in fruits and seeds as free cytokinins and has been suggested that many of the physiological processes associated with seed development are controlled by these dihydro cytokinins (Van Staden, 1983).

The second cytokinin encountered, appeared after 6 and 8 weeks of cold stratification of both the testa and mesocarp. Its Rf(0.25) came very close to those of BAP marker (Rf=0.23). Its appearance at the sixth and eight weeks of stratification could be inferred as the increase of this BAP-like cytokinin as a consequence of protracted chilling and it is speculated that its level could be higher at the tenth week, resulting in the after-ripening of the embryos. Apparently, it did not appear at any period at the embryos or if it does,it may be at the 8^{th} or 10^{th} week and its presence would be in very minute quantity rendering it undetectable. If this hypothesis is true, it could be that this BAP-like cytokinin is produced in both the seedcoats and protracted chilling triggers its production that could tilt the promoter-inhibitor balance. It may also not be detected in the embryos because CK, besides its minute quantity, is apparently not exported readily (Van Staden, 1983) but that it could be metabolised rapidly and extensively within these organs (Davey and Van Staden, 1981).

In one of the few studies available on this species, Hilton *et al* (1965) reported that the eluates from Rf 0.8 - 0.9 of chromatographic (paper chromatography with isopropanol-water (8 : 2) solvent) extracts of dormant *S. aucuparia* seeds had a marked inhibitory effect on the elongation of wheat coleoptiles and repressed seedlings of two woody species. These inhibitor or inhibitors in *S. aucuparia* seeds are yet to be identified. A widely accepted hypothesis that the breaking of dormancy and the subsequent commencement of germination depends on a balance between endogenous growth inhibitors and promoters (Amen, 1968). From the preceeding studies exogenous application of growth promoters, such as BAP has been able to alter this balance thus promoting the after-ripening of otherwise dormant seeds. Or perhaps the concept that has gained growing support (Bradbeer, 1988) that the physiological effects of plant growth substances may result from changes in cellular sensitivity to them (Trewavas, 1982). Therefore, there should be a correlation between the state of dormancy and either the concentration of endogenous inhibitor or the sensitivity of the embryo to the inhibitor.

The identity of perhaps the third chemical with an Rf value of 0.44 obtained from chromatogram developed from *system 2* is not known. It was suggested to be a cytokinin for its staining characteristic to be similar to the CK (BAP and K) standard used.

The fourth chemical (Rf = 0.34) found also in *System 2* has an Rf similar to that of *cis zeatin* (Rf = 0.32) reported in Leonard *et al* (1971). Its presence in the kernels of sweet corn has been identified (Summons *et al* , 1980) and this findings seem to agree to it being detected in the testa of *S. aucuparia* in the current study.

4. MICROPROPAGATION STUDIES OF *SORBUS AUCUPARIA* L.

4.1 Experiments to assess the effects of different combinations and concentrations of plant growth regulators (BAP and IBA) on multiple shoot proliferation in *Sorbus aucuparia* L.

4.1.1 Experiment 1: The Effects of BAP and IBA on excised embryos together with a high percentage of sucrose added to the basal medium

From most excised embryos where the seed coats were removed hypocotyls, roots and shoots were obtained (Figs.IV.19, 20 and 21). About 50 % of the embryos subjected to the various treatments recorded callus formation within the first month. The calli were formed either at the embryonal part of the cotyledon, or occasionally at the edges of the cotyledon. From these callus adventitious roots and 'hypocotyls' were generated and later shoots developed.

No hypocotyl growth was recorded by embryos subject to the control (a1) treatment or for any treatment that did not contain BAP (a2 - a10) (Fig. IV.19a). The embryos remained dormant, retaining a creamy greenish coloration.

Hypocotyl growth, as measured by an increase in length was observed in many of the treatments where BAP and IBA were present in combination (Figs. IV.19a, b, c). In particular, BAP at a concentration of 0.2 mg/l and 2 mg/l with IBA at 0.05 mg/l and 0.1 mg/l respectively produced the greatest hypocotyl growth (Figs IV.19a, c).

Overall, however, many of the embryos remained dormant with only 31 % showing signs of growth (165 out of 540 embryos).

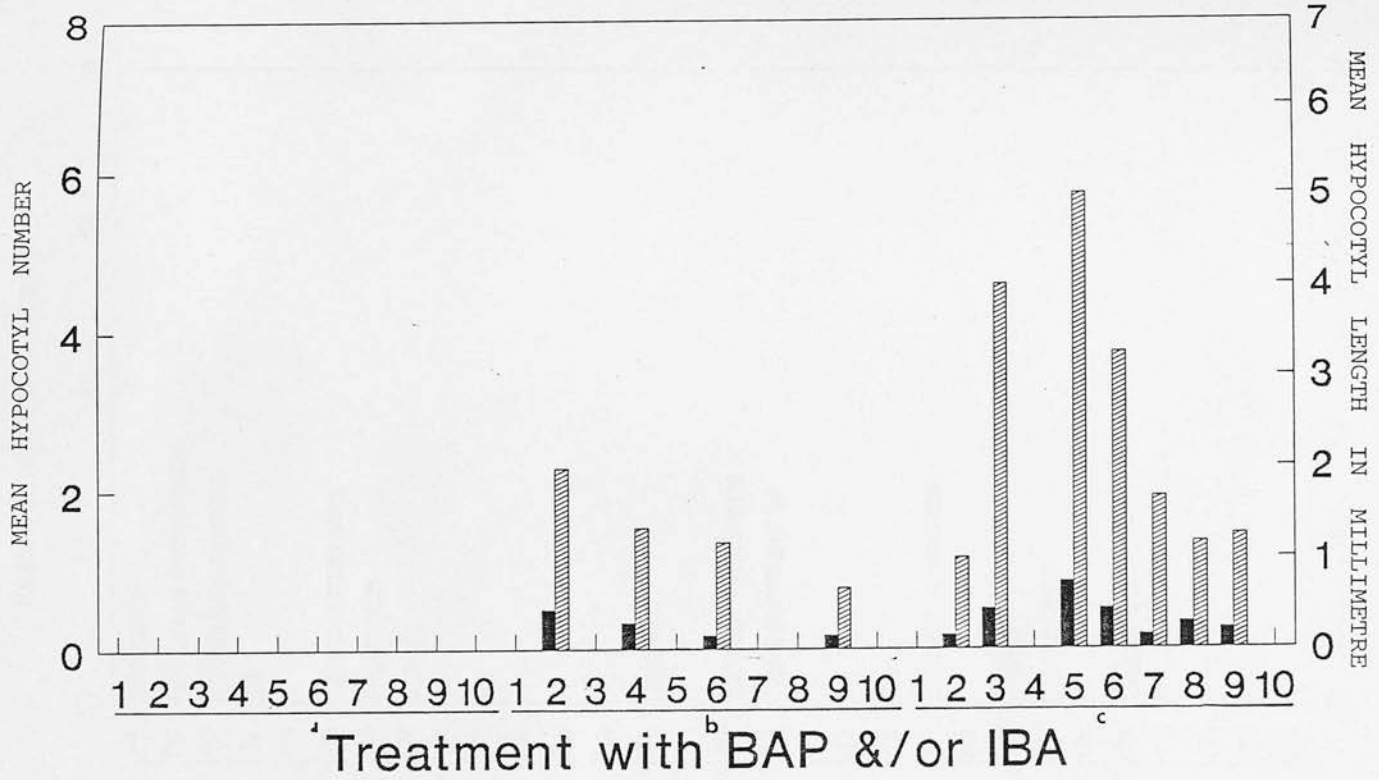


Fig. IV.19a: Hypocotyl Number and Hypocotyl Length relationship with increasing levels of BAP &/or IBA after 1.5 months of culture (for explanation of the codes see Table III.2)

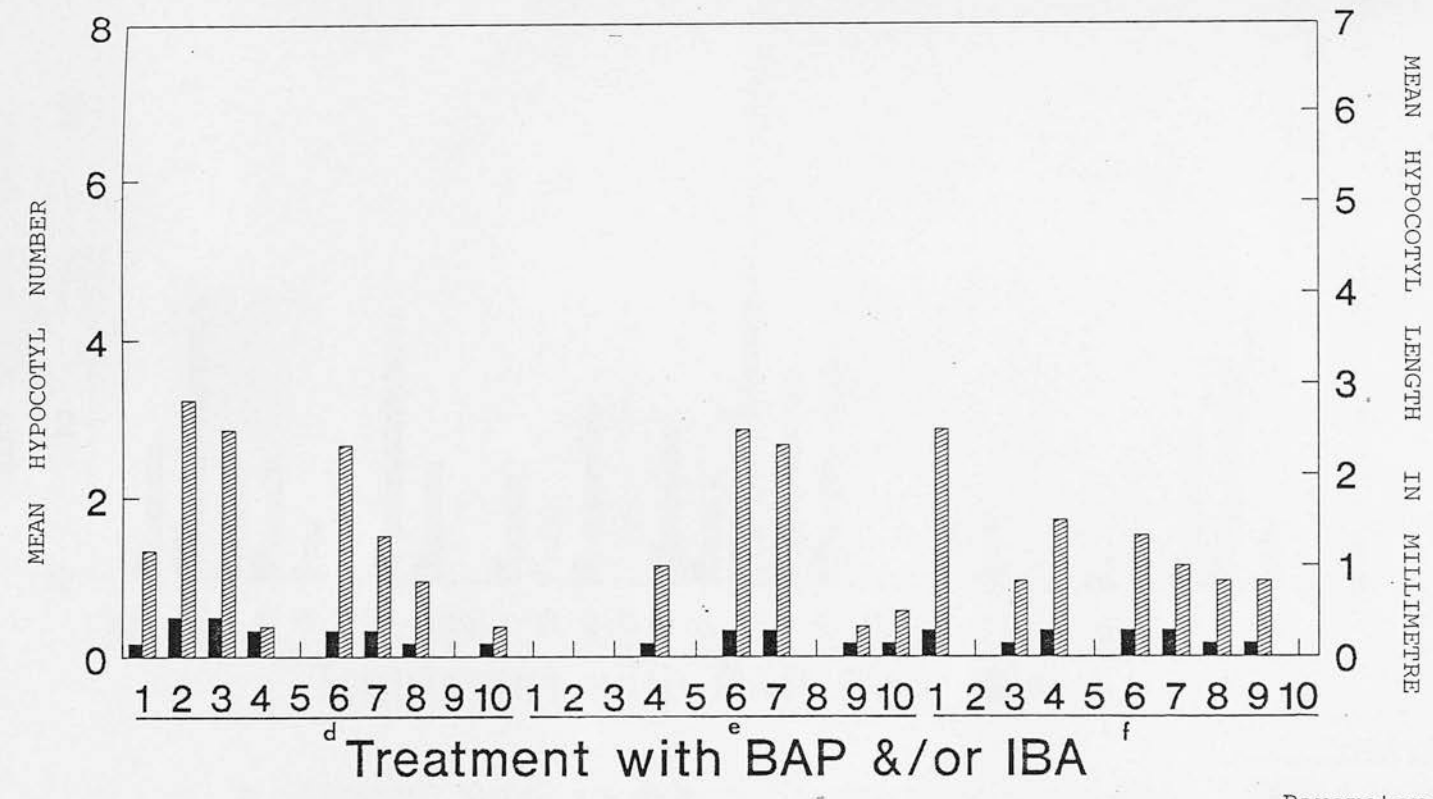


Fig. IV.19b: Hypocotyl Number and Hypocotyl Length relationship with increasing levels of BAP/IBA after 1.5 months of culture (for explanation of the codes see Table III.2).

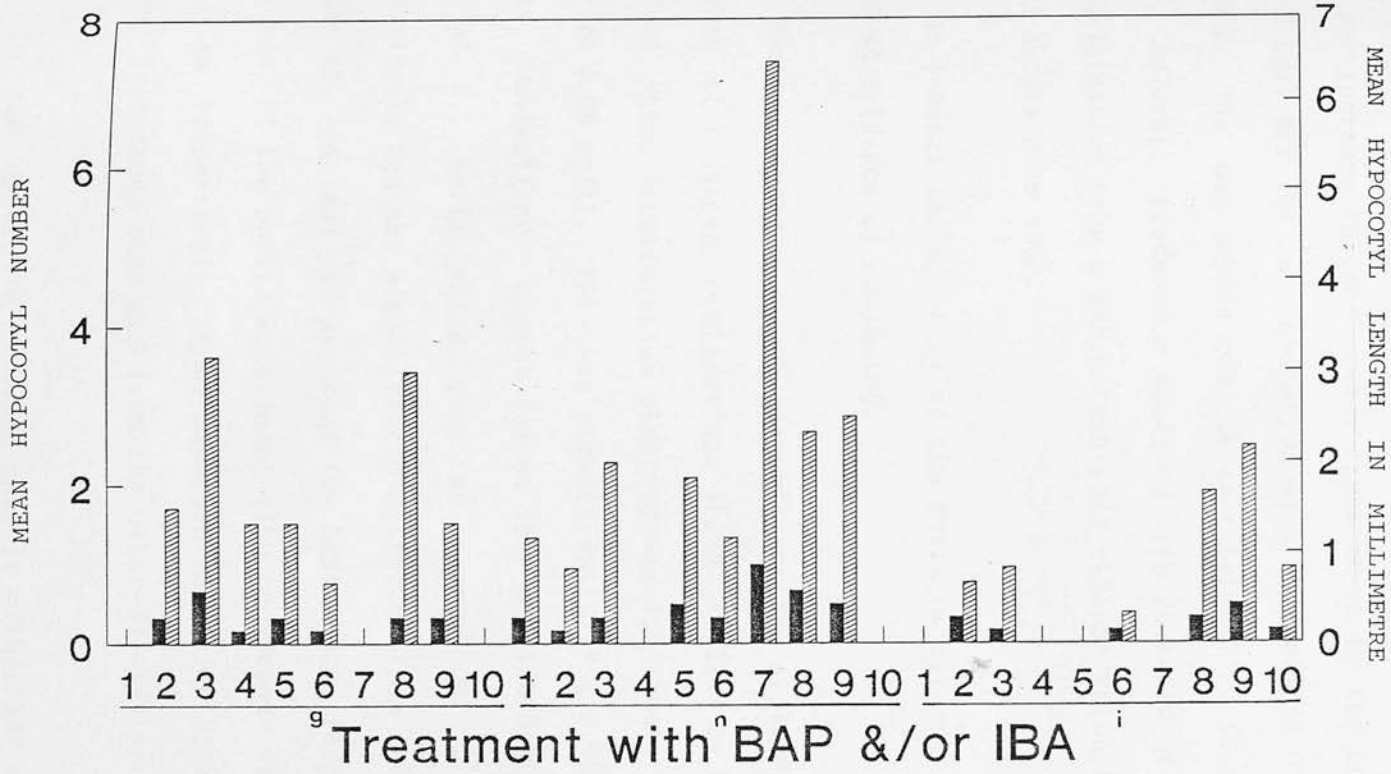


Fig. IV.19c : Hypocotyl Number and Hypocotyl Length relationship with increasing levels of BAP &/or IBA after 1.5 months of culture (for explanation of codes see Table III.2)

Root growth was also promoted by the various hormonal treatments. In particular, root length was promoted either by BAP at 0.2 mg/l in combination with IBA between 0.05 - 0.1 mg/l or BAP (0.4 mg/l) in combination with IBA in a range between 0.04 - 0.1 mg/l (Figs.IV.20a, b). An increase in root number appeared to be the result of treatment with 2 mg/l BAP (h) in combination with IBA between 0.05 - 1 mg/l (Fig. IV.20c). The one embryo subject to treatment h9 (2 mg/l BAP/0.5 mg/l IBA), however, produced a seedling with 20 roots of average length 8.5 mm, originating from a large "embryoid callus" which formed a rosette of dwarf shoots 2 mm high.

In general the majority of the cultures subject to treatment series h showed evidence of callusing.

Shoot production, both adventitious and axillary , was recorded in a range of treatment combinations (Figs. IV.21a, b, and c). The highest average shoot proliferation observed was 3.5 shoots in medium c6 (BAP 0.2/IBA 0.08 mg/l). This was significantly ($P \leq 0.001$) better than all other combinations, except for c5 (BAP 0.2/IBA 0.05 mg/l) and d7 (BAP 0.4/IBA 0.1 mg/l) which gave an average of 3 and 3.33 shoots respectively and not significantly different from c6 above. Similarly, media c5, c6 and d7 promoted the best shoot length growth of the explants of the entire experiment with an average of 13.43, 13.70 and 12.37 mm. respectively. These averages were significantly different ($P \leq 0.001$) from those obtained from the other 83 treatments.

It was generally observed that if either BAP or IBA was present alone no shoot, root or hypocotyl growth occurred (Table: IV.13; Figs. 19a, 20a, 21a (a2 - a10)).

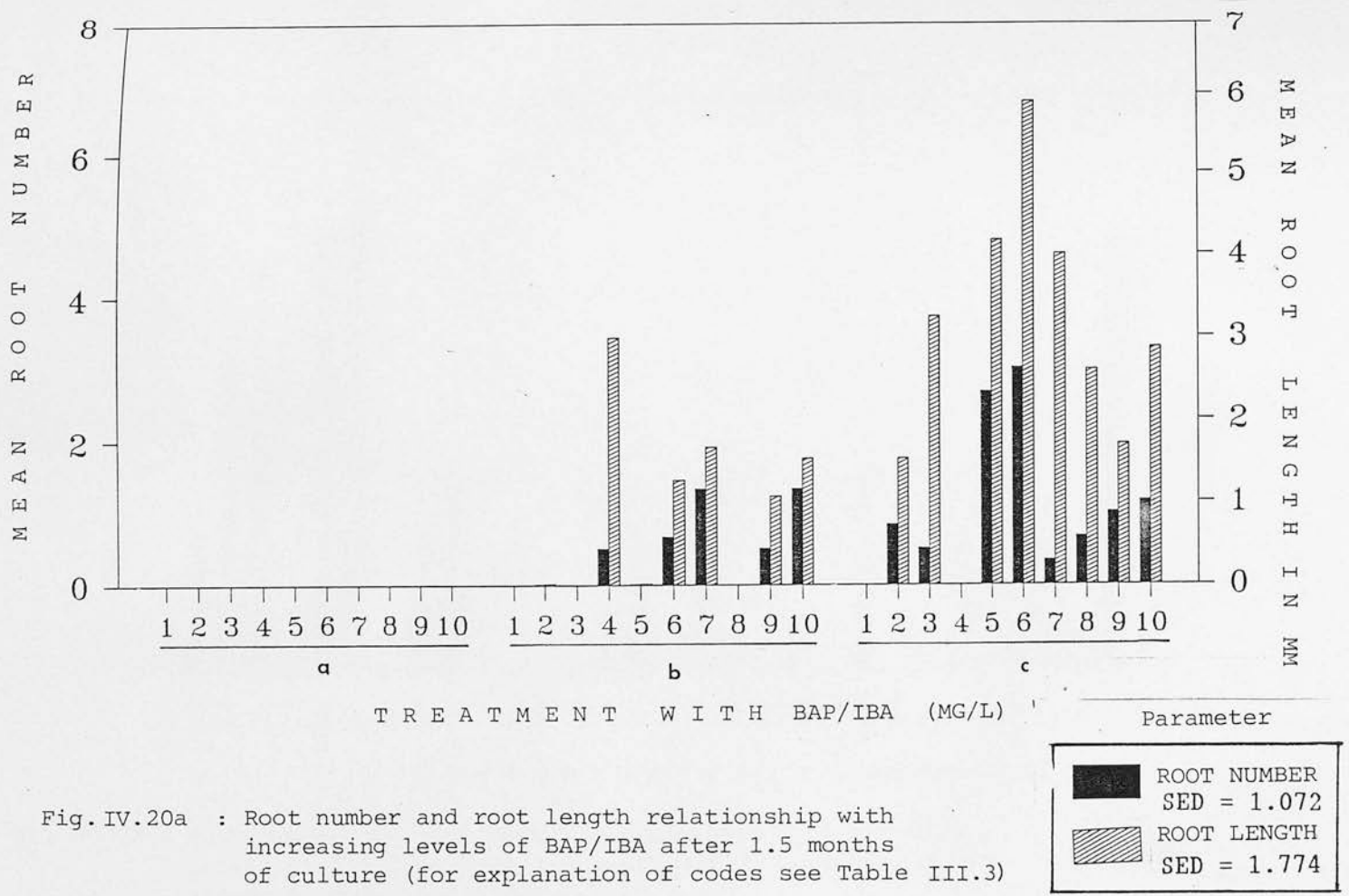


Fig. IV.20a : Root number and root length relationship with increasing levels of BAP/IBA after 1.5 months of culture (for explanation of codes see Table III.3)

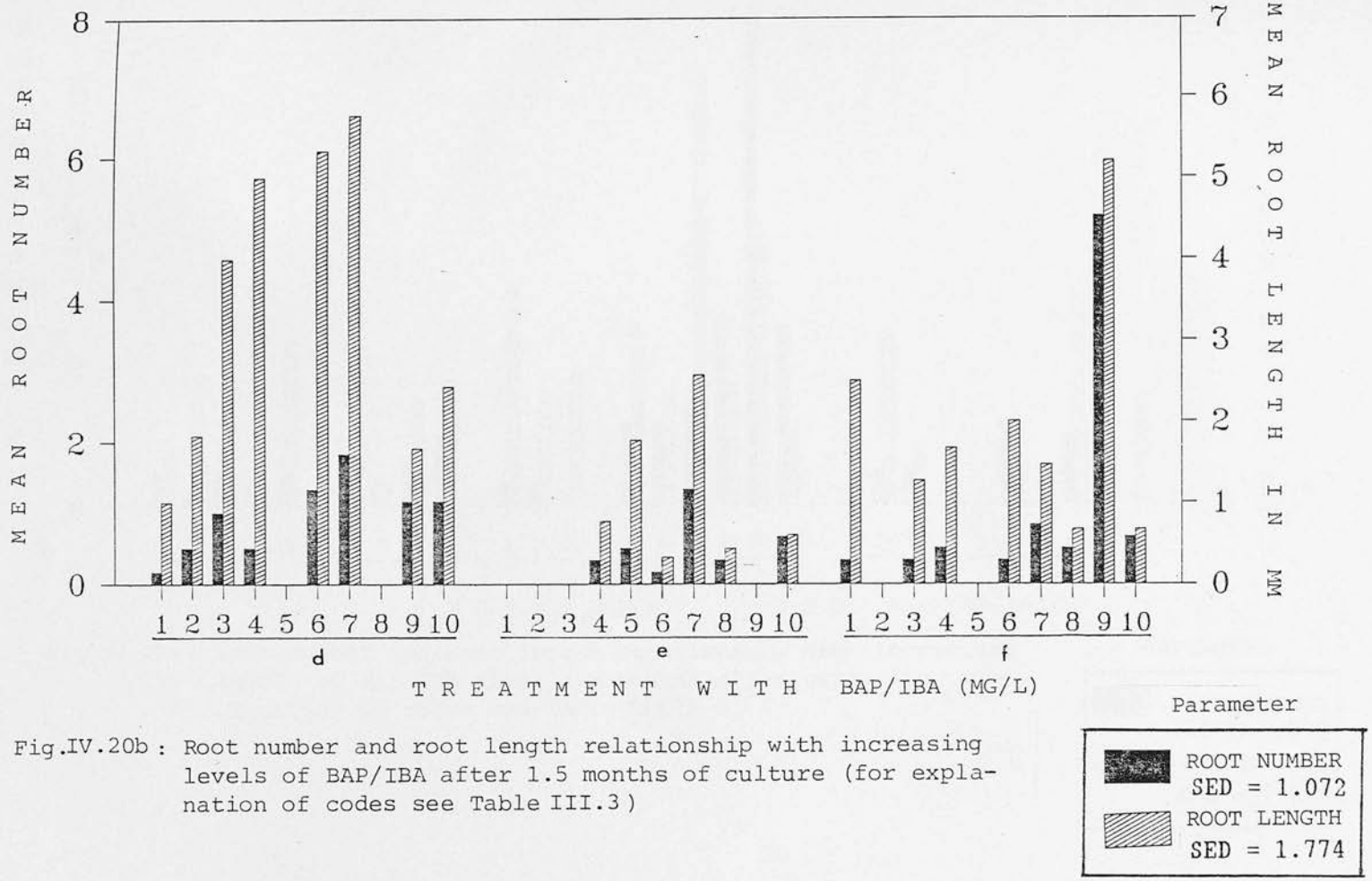


Fig.IV.20b : Root number and root length relationship with increasing levels of BAP/IBA after 1.5 months of culture (for explanation of codes see Table III.3)

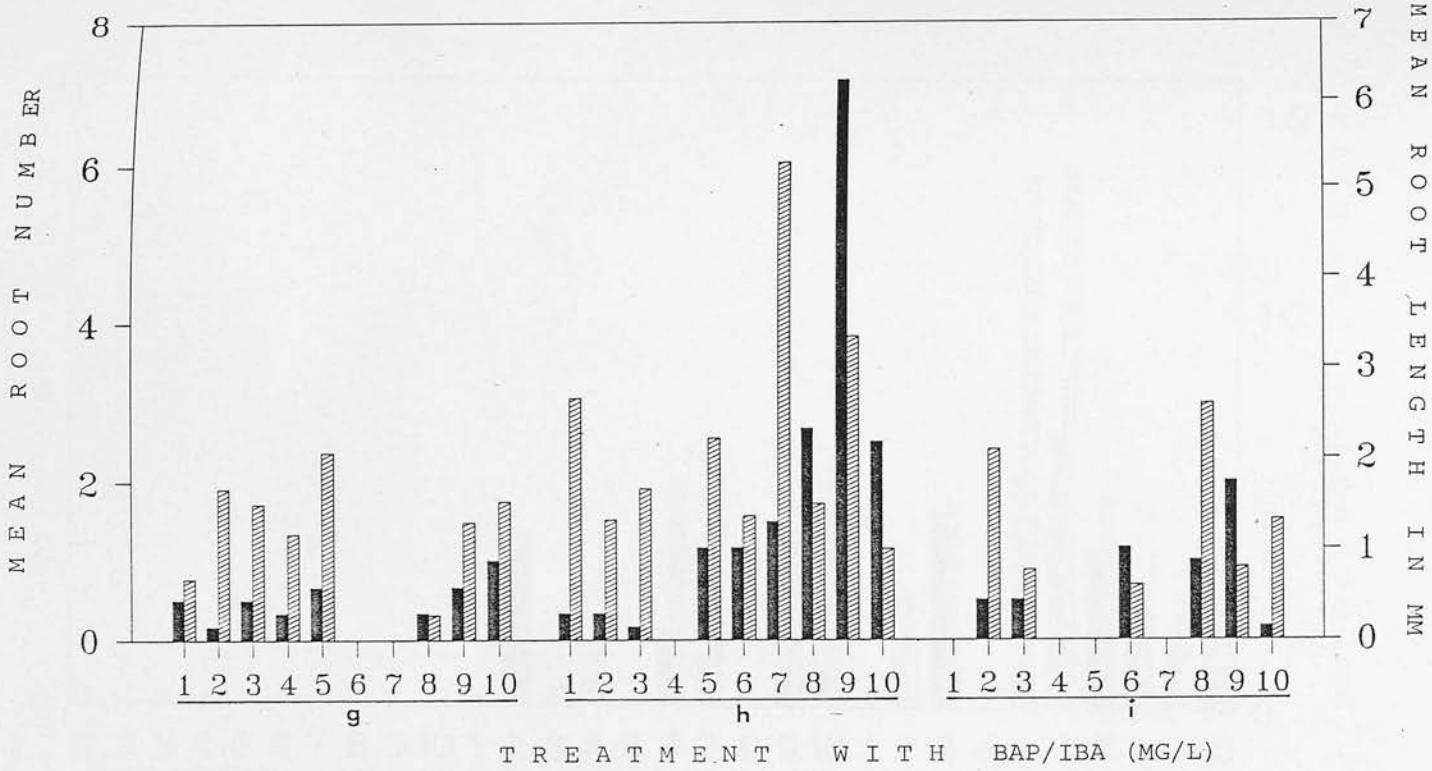


Fig.IV.20c : Root number and root length relationship with increasing levels of BAP/IBA after 1.5 months of culture (for explanation of codes see Table III.3).

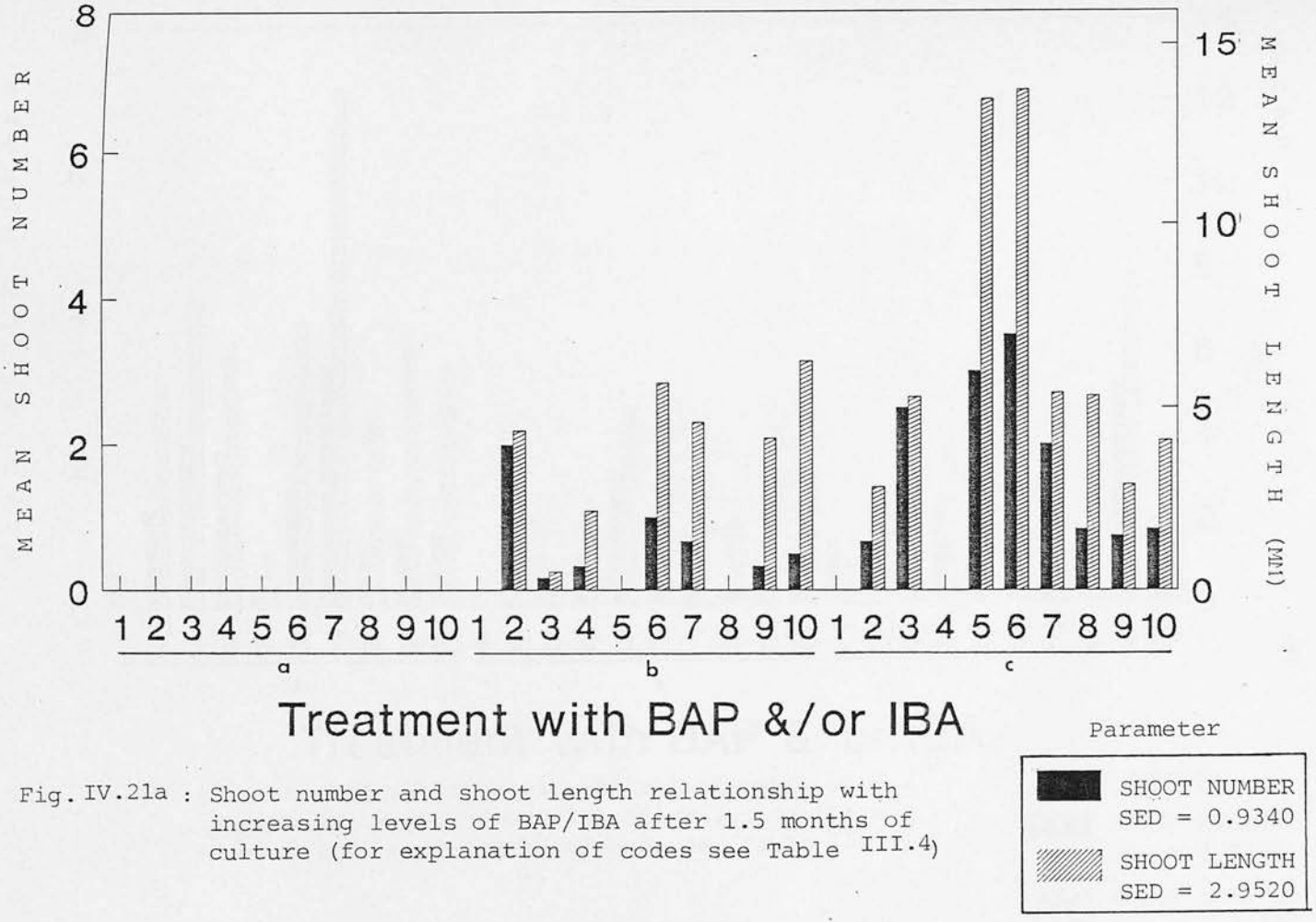


Fig. IV.21a : Shoot number and shoot length relationship with increasing levels of BAP/IBA after 1.5 months of culture (for explanation of codes see Table III.4)

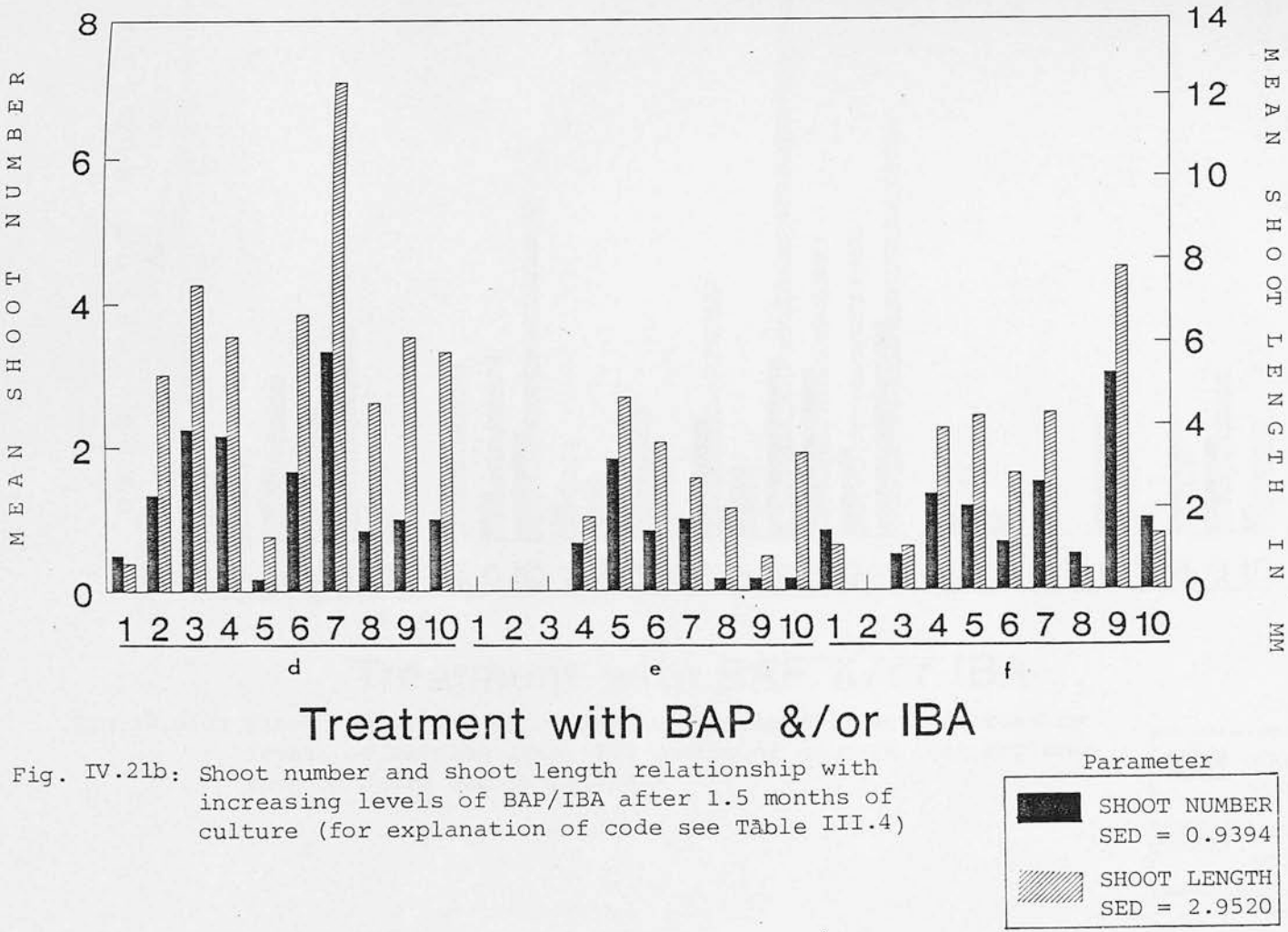


Fig. IV.21b: Shoot number and shoot length relationship with increasing levels of BAP/IBA after 1.5 months of culture (for explanation of code see Table III.4)

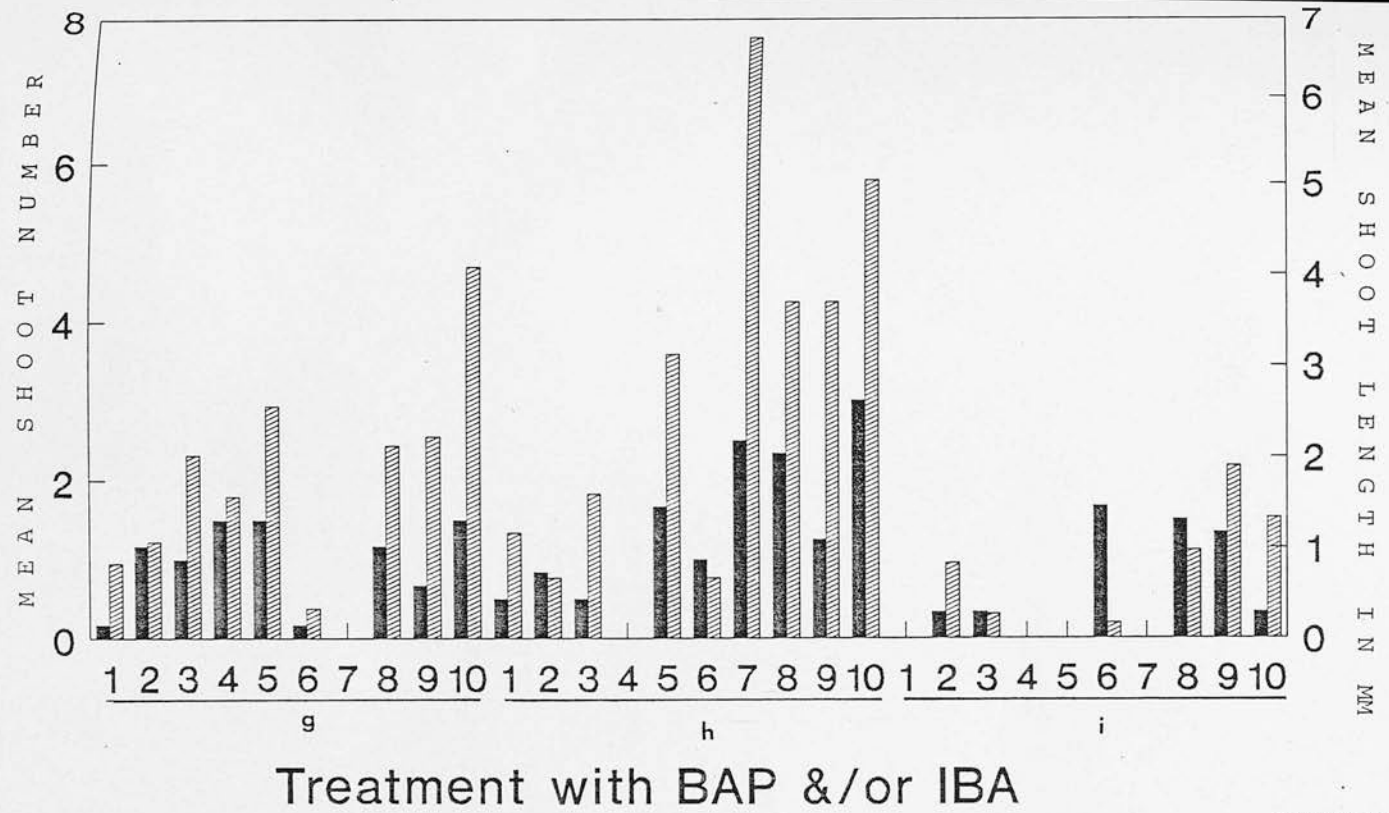


Fig.IV.21c: Shoot number and shoot length relationship with increasing levels of BAP/IBA after 1.5 months of culture (for explanation of codes see Table III.4)

Table IV.13 : Average number of shoot, root and hypocotyl produced by BAP alone on excised embryos of rowan.

BAP(mg/l)	Shoot	Root	Hypocotyl
0	0	0	0
0.1	0	0	0
0.2	0	0	0
0.4	0.5	0.167	0.167
0.6	0	0	0
0.8	0.833	0.333	0.333
1	0.167	0.5	0
2	0.5	0.333	0.333
5	0	0	0

4.1.2 Discussion

Excised embryos of *Sorbus aucuparia L.* were stimulated to produce hypocotyls, roots and shoots, when both cytokinin (BAP) and auxin (IBA) were present in the growth media at different concentrations. Multiple shoots originated at the cotyledonary node region with or without callus whereas root formation in cultures was preceded by a callus stage,more roots were formed the longer the cultures were incubated. From this experiment it was clear that there existed a sharp sensitivity of the rowan embryo to the range of growth regulator concentrations tested. The best level of BAP and IBA concentrations that stimulated multiple shoots and roots were in the region of 0.2 mg/l BAP in combination with 0.05 or

0.08 mg/l IBA. However,the number of embryos that were not stimulated by any combinations of hormonal concentrations was high (31 %) and may suggest that there is a high degree of variability in the depth of dormancy in rowan and that exogenous BAP + IBA are only effective at the less deep stages of dormancy.

The shoots, roots and hypocotyls were adventitious originating from callus, however, this could be confirmed by sections and examination under the light microscope.

As far as is known, this study is the first report of rowan shoots and roots regenerated from excised embryos by tissue culture techniques. Jorgensen and Binding (1983) reported that they germinated rowan seeds in culture media MS (Murashige and Skoog, 1962) with 5 µM BA(1.13 mg/l), or in B5 (Gamborg *et. al.*, 1968) with 2.5 µM BA (0.56 mg/l). Kouider *et.al.* (1984) also found that excised embryos of cv Red Delicious apple produced callus and adventitious shoots when they were cultured in a media supplemented with 4 mg/l of BAP.

4.2 Experiment to investigate the influence of cytokinin and auxins on shoot multiplication and elongation

4.2.1 Experiment 2a: Shoot multiplication and elongation in the initial experiment

Control explants appeared green at the time of recording but no shoot growth was apparent.In general nodal segments cultured from the 8 month-old seedlings on media with hormones added, proliferated shoots within three weeks of culturing on the various media. Shoot growth occurred in

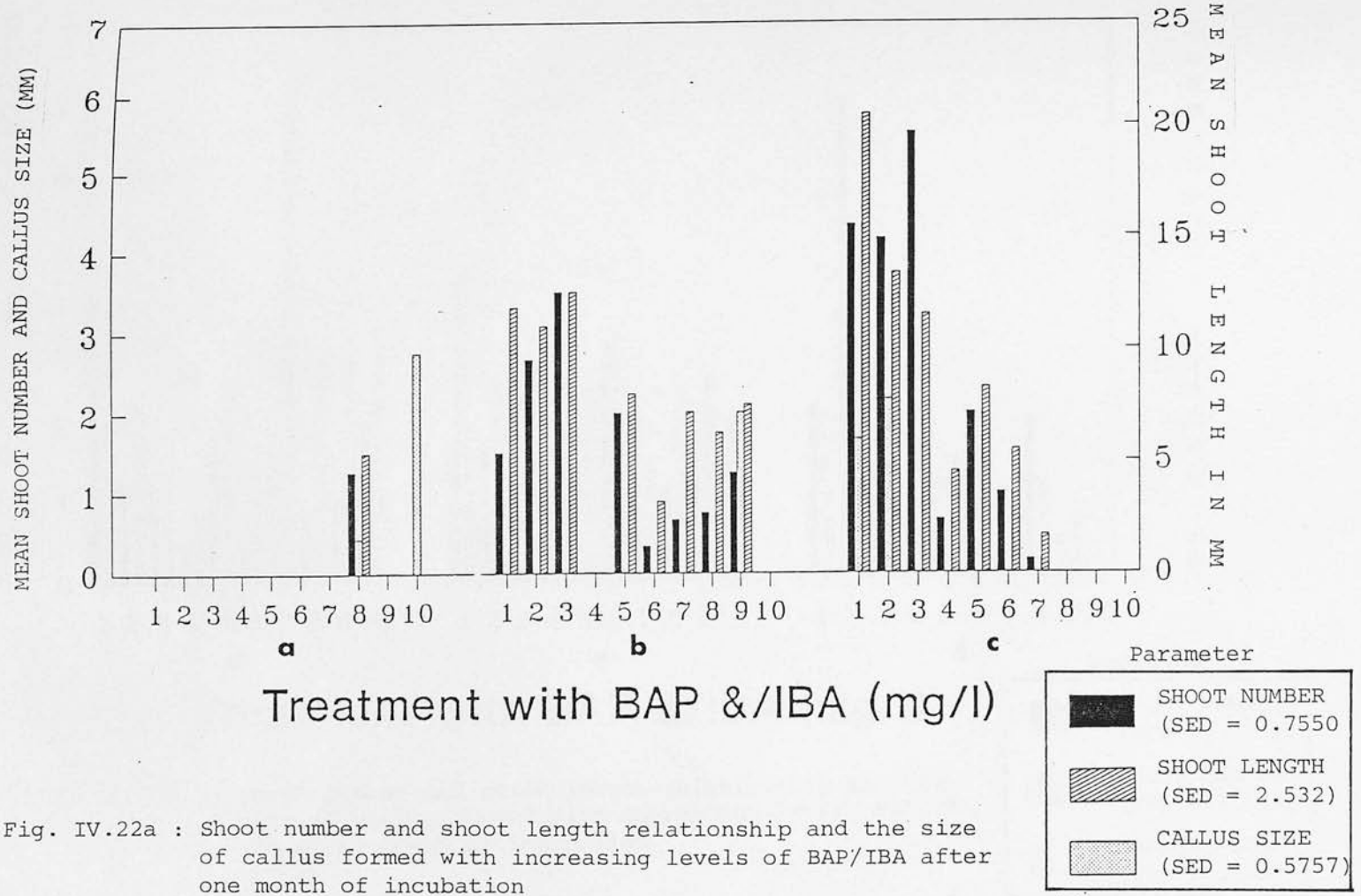


Fig. IV.22a : Shoot number and shoot length relationship and the size of callus formed with increasing levels of BAP/IBA after one month of incubation

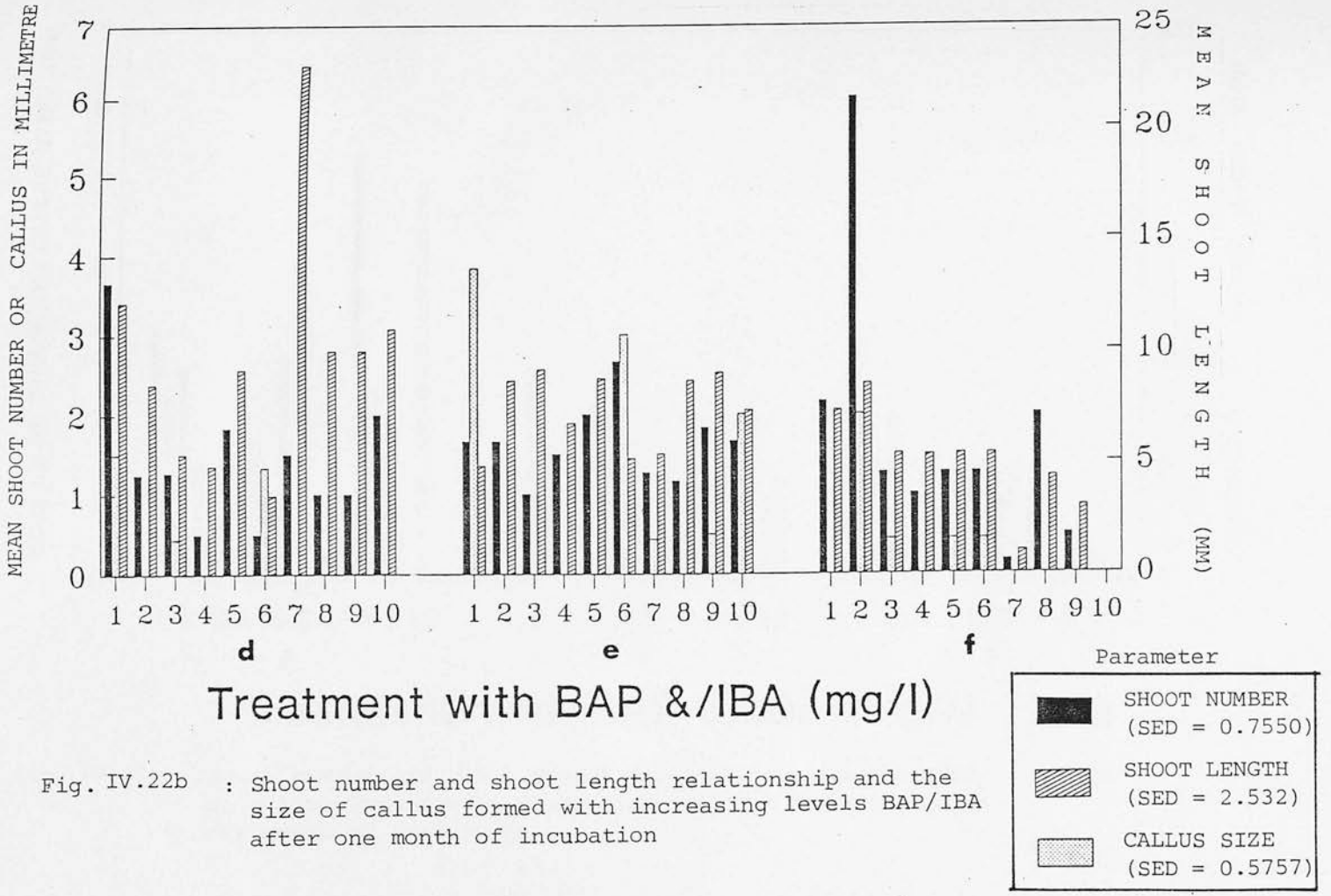


Fig. IV.22b : Shoot number and shoot length relationship and the size of callus formed with increasing levels BAP/IBA after one month of incubation

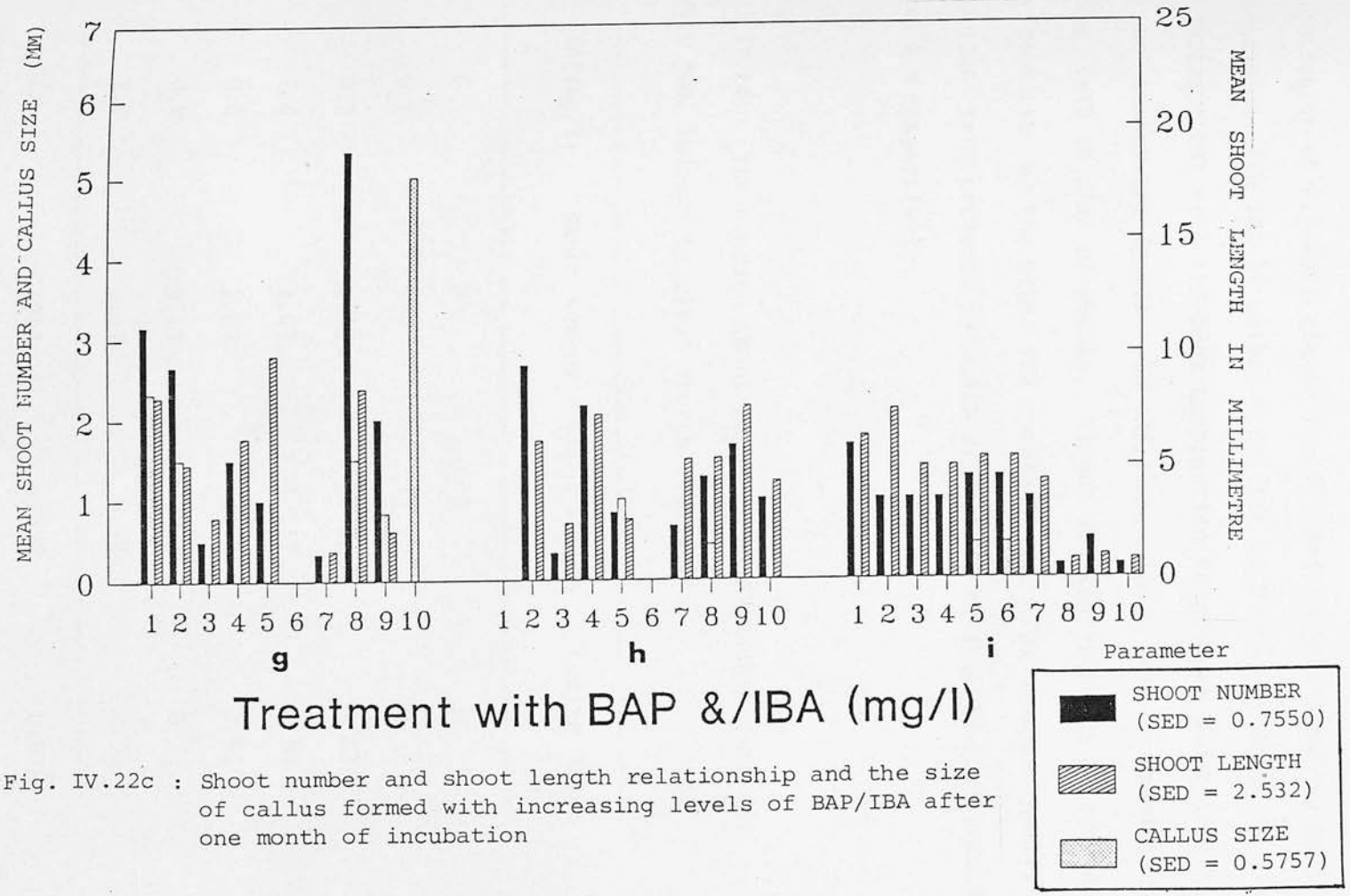


Fig. IV.22c : Shoot number and shoot length relationship and the size of callus formed with increasing levels of BAP/IBA after one month of incubation

all cultures supplemented with BAP(Fig IV.22a and b) but cytokinin-free medium did not effect any shoot growth (Fig.IV.22a,series-a). A medium supplemented with BAP alone produced good multiple shoots, especially at a concentration of 0.2 mg/l (Table IV.14). Media containing 0.2 mg/l BAP in combination with IBA at either 0.01 (Fig IV.22a, c2) or at 0.02 mg/l (Fig IV.22a, c3) were the best combination to promote shoot growth in terms of number and length. In addition, they were morphologically superior both in size of shoots, vigour and the greening of leaves to those produced by the other PGR combinations. The average number of shoots that were produced by media c1, c2 , c3 (Fig.IV.22a) were 4.3, 4.2 and 5.5 respectively.

Table IV.14: The average shoot number, shoot length (mm) and callus diameter (mm) induced by plant growth regulator.

BAP(mg/l)	Shoot Number	Shoot Length	Callus Size
0	0	0	0
0.1	1.5	11.83	0
0.2	4.33	20.50	1.67
0.4	3.67	12.17	1.50
0.6	1.67	4.83	3.83
0.8	2.17	7.33	0
1.0	3.17	8.17	2.33
SED	0.755	2.532	0.576

Analysis of variance indicated that these three media had a significant effect on shoot proliferation ($P < 0.01$).In addition, the average shoot length obtained from media c1 (20.50 mm) was significantly longer than those produced by media c2 (13.33 mm) or c3 (11.50 mm). The medium that produced the highest average shoot number(6) was f2 (Fig.IV.22b); a combination of 0.8 mg/l BAP and 0.01 mg/l IBA. However, the shoots had a lower average shoot length (8.5 mm). In general, within the studied concentration range, shoot production was inhibited by higher IBA concentrations. In addition, at higher concentrations of IBA shoot proliferation and shoot length were inhibited with some concentrations of BAP (which were 0.1, 0.2, 0.8, 2 and 5 mg/l)compared to explants growing on lower IBA concentrations (Figs.IV.22a,b and c).

About 9 % of explants were contaminated out of a total of 540 used. In addition, some explants became chlorotic after one or two weeks of growth. Callus formation occurred in about 10 % of the explants (Fig IV.22a and b), however, its occurrence was sporadic and distributed across all treatment combinations. There seemed to be no general trend of callus formation, however, it occurred with greater frequency in auxin-free media.

4.2.2 Experiment 2b: Shoot multiplication and elongation in the second experiment

Shoot growth occurred within two weeks of inoculation, in nodal and shoot tips cultures derived from two month-old shoots. All media supplemented with BAP stimulated rapid growth of multiple shoots (Fig IV.23, Plate IV.8).In general, as BAP concentration were increased more shoots were formed. BAP alone either at 0.1 mg/l (c1) or 0.15 mg/l (d1)

mg/l or 0.2 mg/l (e1) were most effective (Fig IV.23), at 0.3 mg/l BAP (f1) multiple shoot production was less, although this was not statistically significant. In addition, however, the supplementation of a low concentration of IBA to e3 **(0.2 mg/l BAP/0.02 mg/l IBA)** or to f2 **(0.3 mg/l BAP/0.01 mg/l IBA)** (Fig IV.23) seemed to enhance shoot proliferation and elongation. The former was the best medium for shoot multiplication producing an average of 6 shoots per explant and the shoots obtained were morphologically superior in appearance (Plate IV.8 and 9).

In media supplemented with IBA alone 17 % (5 out of 30) of explants produced multiple shoots (average 2.6 shoots per explant), with an average length of 11 mm after 1 month of culture (Fig IV.23, a2, a3 and a4). The presence of a low concentration of IBA in media b2 (0.05 mg/l BAP and 0.01 mg/l IBA) increased the mean shoot production and also more than doubled shoot length (Fig. IV.23). There were significant differences ($P < 0.001$) between mean shoot number produced by media b2 and b1(0.05 mg/l BAP and 0 mg/l IBA), and between b2 and b4(0.05 mg/l BAP and 0.04 mg/l IBA) but not between b3(0.05 mg/l and 0.02 mg/l IBA). There were also significant differences ($P < 0.001$) between the mean shoot length produced by media b2 and that produced by b1,b2 and b4 but not between b2 and b3. Mean shoot length was longest on media b2 (Fig.IV.23), where BAP was the lowest. Shoot length decreased as BAP concentration increased. In general, mean shoot length increased as IBA concentration increased.

Overall, 37 % of the explants showed evidence of callus, its frequency increased as the concentration of BAP increased (Table IV.15).

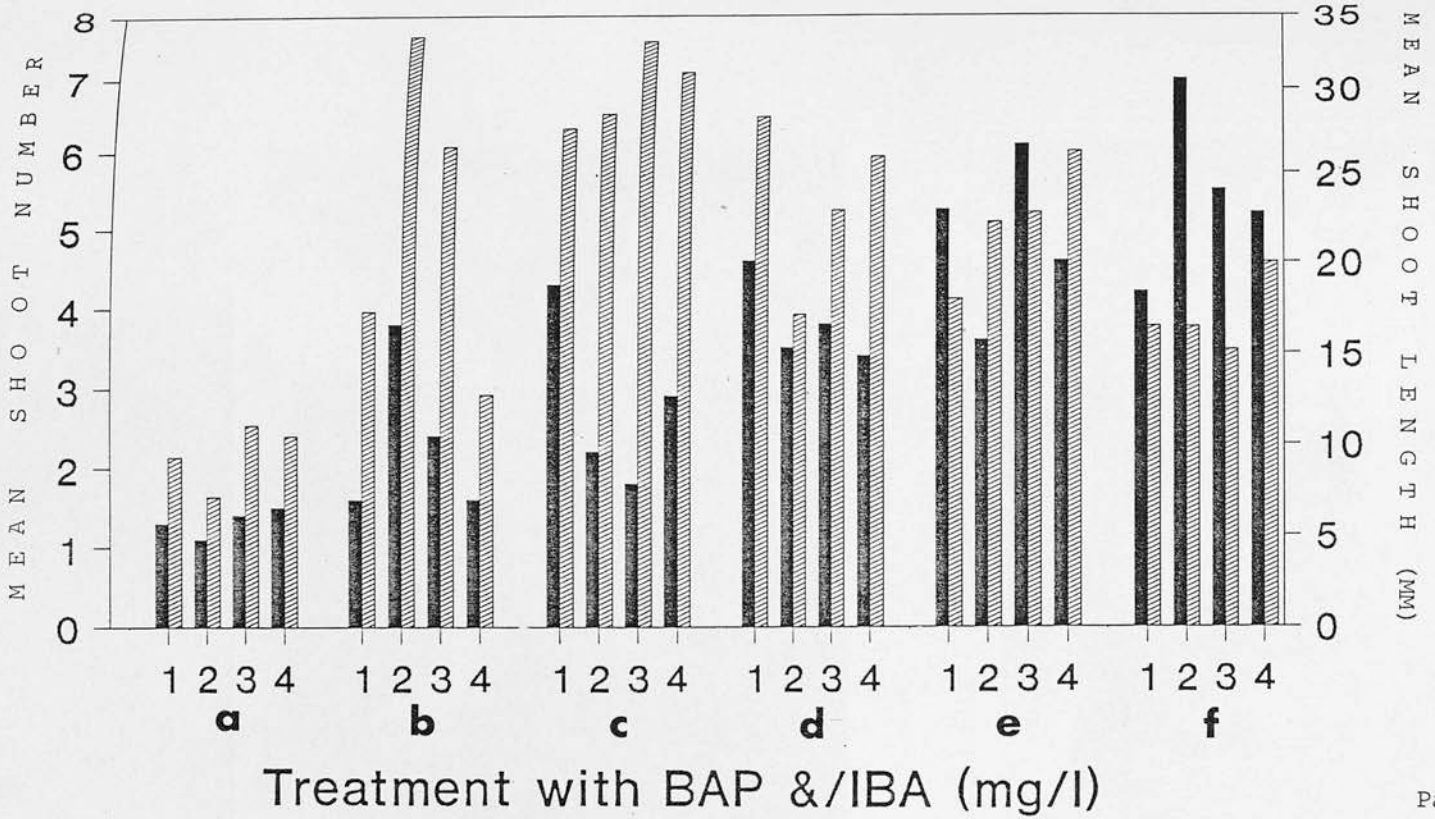


Fig. IV.23 : Shoot number and shoot length relationship with increasing levels of BAP/IBA after one month of incubation

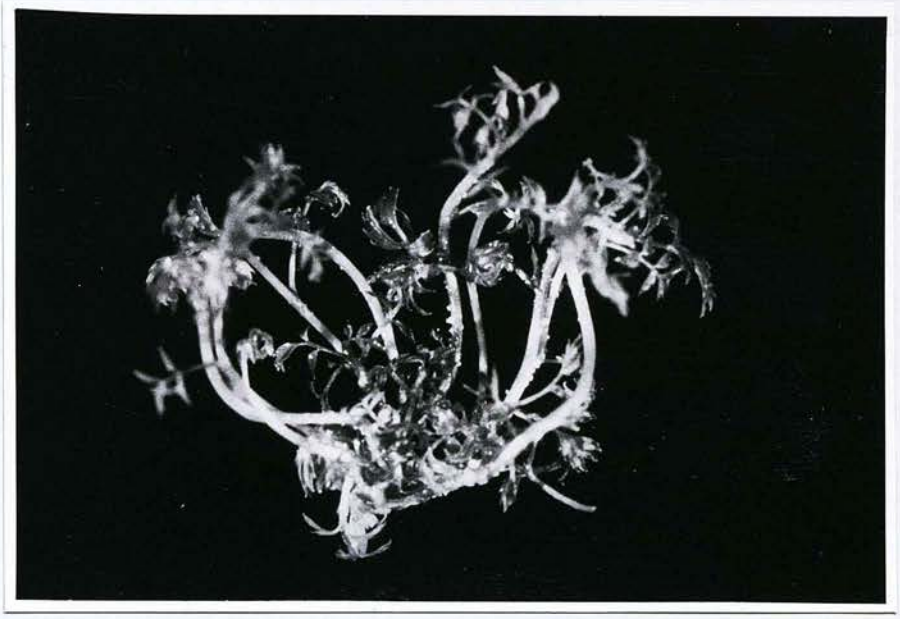


Plate IV.8 : Nine multiple shoots generated in e3 medium (0.2 mg/l BAP + 0.02 mg/l IBA)

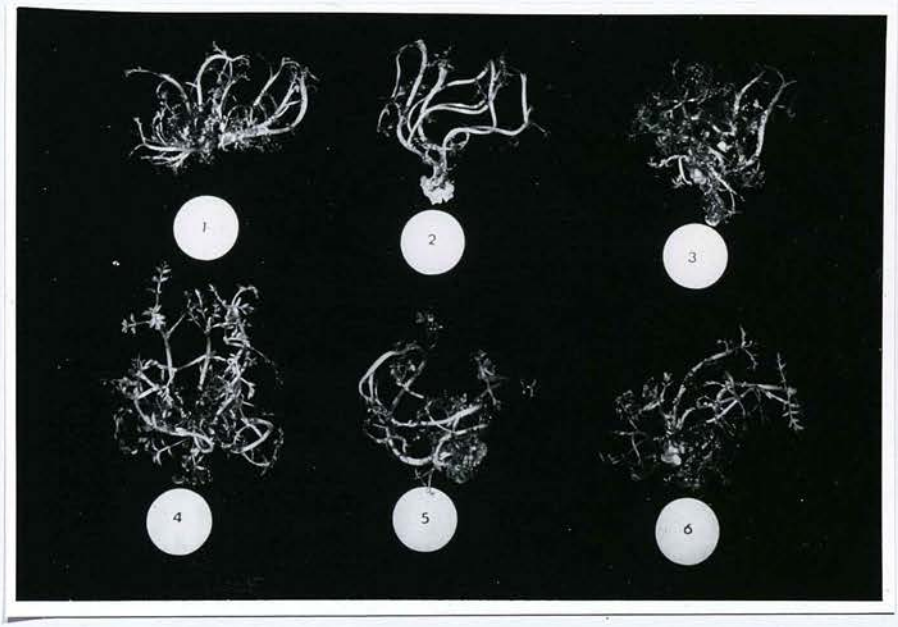


Plate IV.9 : Shoot proliferation of cultured nodal and shoot tip explants derived from 2 month-old shoots on half strength MS basal medium supplemented with 3% sucrose, 0.6% agar and various combinations of CK and auxin (BAP + IBA) as follows;

1. 0.1+0.04 mg/l (medium c4) 2. 0.15+0.01 mg/l (medium d2)
3. 0.2+0.01 mg/l (medium e2) 4. 0.2+0.02 mg/l (medium e3)
5. 0.3+0.01 mg/l (medium f2) 6. 0.2 mg/l BAP (medium e1)

Table IV.15 Percentage of explants that produced callus in the different media tested for shoot proliferation of two-month old shoot materials

Percentage of callusing (%)

Codes	BAP	a	b	c	d	e	f	Total no of Explants
IBA	Concentration (mg/l)	0	0.05	0.10	0.15	0.20	0.30	
1	0	0	0	10	10	10	20	4
2	0.01	0	10	0	0	0	60	7
3	0.02	0	0	20	0	10	50	8
4	0.04	0	0	0	30	80	70	18
Total number of explants		0	1	2	4	10	20	37(37%)

Table IV.16: Biomass indices[1] as an indicator of performance of shoot proliferation in various media in Experiment 2b employing 2-month old shoot explants

BAP (mg/l)	0	0.05	0.10	0.15	0.20	0.30
IBA (mg/l)						
0	12	28	119	130	95	69
0.01	8	127	63	60	80	115
0.02	16	64	60	87	139	84
0.04	16	20	90	88	121	104

1. Index of biomass were obtained by multiplying average number of shoot produced by the average shoot length.

Media e4, f2, f3 and f4 showed particularly high levels of callus both in terms of the number of explants and its intensity. These four media account for more than 70 % of callus encountered in the whole experiment.

Shoots subject to the control treatment survived and appeared healthy with no apparent increase in height but the leaves were observed to be growing. In addition, 10 % of explants in the control treatment produced three roots with an average length of 38 mm and 30 % produced 2 shoots of average height 16 mm.

The highest index of biomass was 139 (Table IV.16) obtained from media e3 (BAP 0.20 mg/l and IBA 0.02 mg/l), this was followed by an auxin free medium supplemented with only BAP 0.15 mg/l. From this it appeared that e3 was the most superior, in that it produced plantlets with multiple shoots of a good length with little callus growth. However, auxin free medium of BAP alone seem to fare very well especially media c1 and c2.

4.2.3 Discussion

Shoot tip and nodal cultures have been found to be a successful method to propagate several different species of broadleaved trees. A range of media have been used for different species. Many workers have found MS medium satisfactory (e.g. Mascarenhas *et.al.* 1982, for *Eucalyptus* and *Dalbergia* ; Vieitez *et.al.*,1988 for Chestnut (*Castanea spp*). Juvenile explants usually begin to grow and proliferate without delay, but explants from mature parts of trees may not start to multiply until they have been cultured for 6 - 10 months (McComb and Bennet, 1982). It has

also been reported in Paily and D'Souza (1986) that shoot multiplication on explants from juvenile, non-flowering (4 year-old) and mature, flowering trees (30 year old) of *Lagerstroemia flos-regia* were dependent on the type and concentration of cytokinin employed. He found that the artificial cytokinin BAP was the best for shoot multiplication. Keys and Cech (1981, 1982) successfully cultured the excised embryos of chestnut (*Castanea dentata L*) to produce axillary shoots and plantlets in MS medium with 1 mg/l BAP added to it.

The present study demonstrates clearly that BAP was successful in inducing shoot proliferation from nodal segments of *Sorbus aucuparia L.* The shoots produced can be further excised from the plants and recycled on proliferation medium e3 (Fig.IV.23) where they grow best. Nodal segments from aseptically germinated 8 month old seedlings grown on MS media with BAP at 0.2 mg/l yielded high numbers of shoots. For all cultures , even small concentrations of CK generally promote the formation of shoots. Better growth of 8-months old shoots was obtained when BAP at 0.2 mg/l and was in combination with IBA at either 0.01 or 0.02 mg/l. This phenomena has been highlighted by Jones (1985) in that IBA acted to enhance the effect of BAP at certain concentrations.

Athough shoots and roots can be generated *in vitro* from excised embryos on a medium containing BAP 0.2 mg/l and IBA 0.05 mg/l (see Experiment 1), however, the roots and shoots were interlocked with each other making it impossible to separate each individual plantlet. Therefore rooting needs to be tried with individual shoots by excising aseptically and explanted them in a culture medium suitable for root

initiation. From shoots obtained from Experiment 2a, it was observed that cytokinin-free medium supplemented with very low concentration of IBA were able to initiate the shoot to produce roots without forming multiple shoots. It was therefore, decided to investigate further by repeating the treatments with more replications. Other methods of root induction were also assessed looking for the possibility of initiating healthy adventitious roots production without callus as well as maintaining growth of the plantlet.

4.3 Experiment to investigate the influence of auxins and a hormone rooting powder on rooting formation of excised shoots.

4.3.1 Experiment 1: The influence of IBA in either a high or a low sucrose medium on root formation on excised shoots

Sucrose level had a marked effect on the rooting performance of excised shoots (Table IV.17 and 18; Fig IV.24a and b). Sucrose at 3 % inhibited the production of roots (Fig IV.24a). No rooting of explants occurred both to controls or explants subject to 0.01 mg/l IBA supplemented in the medium, although they survived, with maintainence of leaf greening. Explants that rooted in media containing 0.2 and 0.3 mg/l IBA, possessed roots that were short with a mean length of 2.8 and 3.3 mm respectively.

In contrast, shoots treated with media containing 0.8 % sucrose produced almost 6 times as many roots compared to explant grown in media containing 3 % sucrose (Plate IV.10)

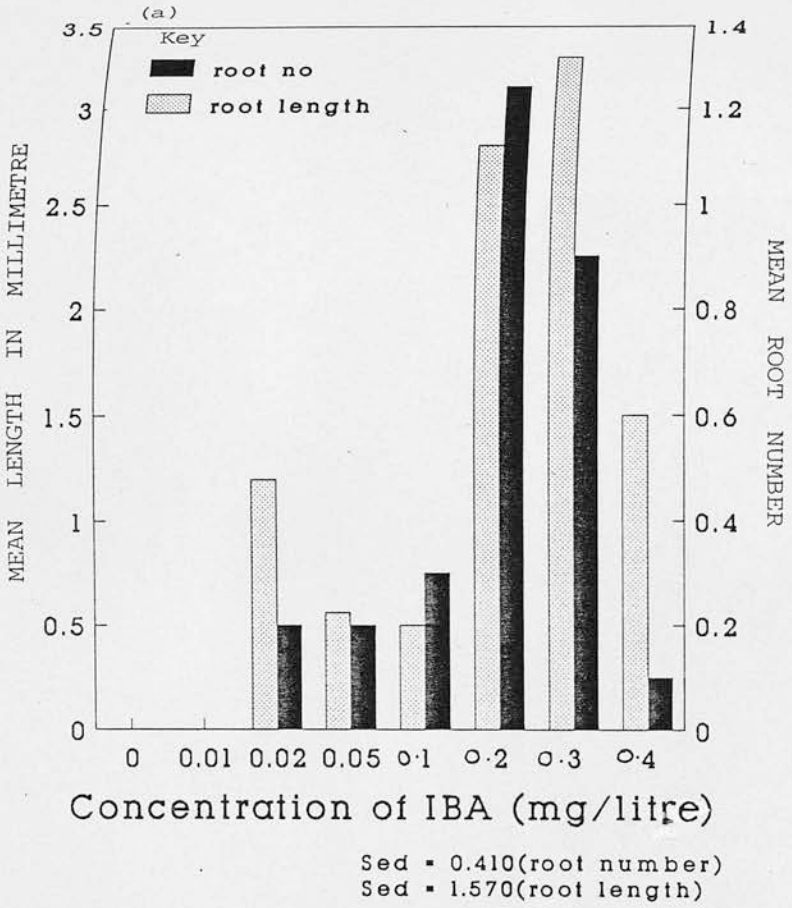
Root production tended to increase as the concentration of IBA increased,there was no significant difference between IBA treatments. However, the control possessed significantly less ($P<0.001$) roots compared to media containing IBA. With IBA concentration up to 0.05 mg/l

Table IV.17: Rooting percentage of excised shoots (n = 10) with and without IBA treatment in media containing 3 % sucrose

Treatment (mg/l)	Total rooted shoots	Total non-rooted shoots	% rooted shoots
Control	0	10	0
0.01	0	10	0
0.02	1	9	10
0.05	1	9	10
0.10	1	9	10
0.20	4	4	40
0.30	4	6	40
0.40	1	9	10

Table IV.18: Rooting percentage of excised shoots (n = 16) with and without IBA treatment in media containing 0.8 % sucrose

Treatment (mg/l)	Total rooted shoots	Total non-rooted shoots	% rooted shoots
Control	14	2	87.5
0.01	16	0	100
0.02	16	0	100
0.04	16	0	100
0.05	16	0	100



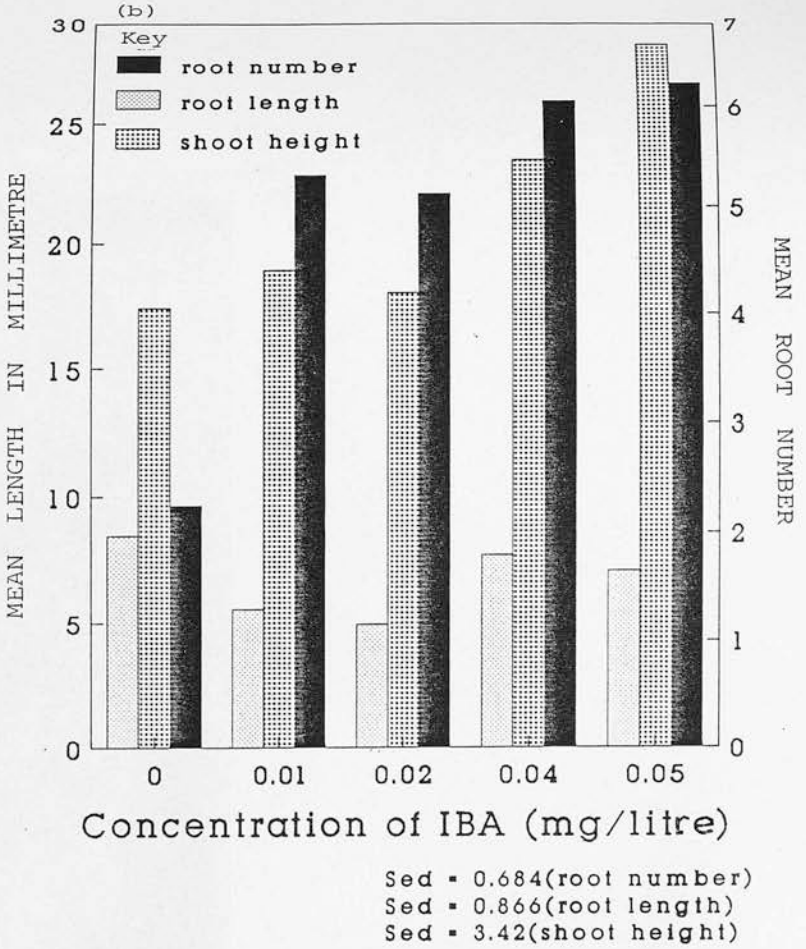


Fig.IV.24 : Effect of IBA on rooting of excised shoots supplemented with (a) 3% sucrose and (b) 0.8% sucrose

Plate IV.10 : Rooting in medium supplemented with 0.04 mg/l IBA

the frequency of shoots forming roots significantly increased compared to controls, at the same time good shoot growth was maintained. Mean root length was not significantly different between treatments, their overall mean being 6.74 mm. Roots were visible after three weeks of incubation.

4.3.2 Experiment 2: The influence of NAA in high sucrose medium on root formation of excised shoots

The results shows that some rooting were observed (Table IV. 19) but there were high incidence of swelling at the root base.

The reduction in the mean values of root production and root lengths measured in the explants grown in media with high sucrose was mainly attributed to the low percentage of rooted explants.

Table IV.19: Rooting percentage of excised shoots (n = 10) with and without NAA treatment in media containing 3 % sucrose added to it

Treatment (mg/l)	Total rooted shoots	Total non-rooted shoots	% rooted shoots
Control	2	8	20
0.01	3	7	30
0.02	0	10	0
0.05	1	9	10
0.10	0	10	0
0.20	3	7	30
0.30	0	10	0
0.40	0	10	0

4.3.3 Discussion

The number and length of roots produced by *Sorbus aucuparia* shootlets in culture increased almost 30 times when the sucrose level was reduced from 3 to 0.8 % with IBA concentrations of 0.02 and 0.05 mg/l respectively. In general, roots grown on an agar medium supplemented with IBA were unbranched and had a distinct morphological appearance in that they were devoid of root hairs and were stout and short in length. However, shoots that rooted in the control media produced normal branched roots with root hairs and with a good root length. However, only a mean number of 2 roots were produced by the control explants (Fig.IV.24b) compared to those treated with IBA which had a mean of 6. Perhaps, a better treatment would be a short root initiation phase in IBA medium followed by transferring the plantlets to a basic medium devoid of PGR to facilitate root elongation.

A phenomenon observed on explants rooted in the presence of IBA was a swelling at the base of each root,not observed in the control. Therefore, this swelling is presumably due to the formation of callus and subsequently the formation of root primordia and can be attributed to the presence of auxin. Auxin enhanced root proliferation but had a deleterious effect on root morphology.

Studies in apples (Jones, 1981; Jans, 1983; Marks, 1985) have shown that the rate of rooting varies with cultivar and that they required between 2 - 6 months of sub-culture before more than 70 % of the shoots were rooted. However, in the present experiments with *Sorbus aucuparia*, cultured shoots were incubated for only 2 months and 100 % of the shoots rooted.

4.3.4 Experiment 3: Influence of combination of auxins on root formation of excised shoots.

Various rooting responses were exhibited by cultured shoots grown in media supplemented with different combinations of IBA/NAA (Fig IV.25). After 4 months culture, rooting was found to be successful in many of the explants (Table IV.20), all combinations of the growth regulators tested were capable of promoting root growth (Table IV.21). There seemed to be no clear and definite rooting response in the entire experiment. However, the degree of callus formed in each treatment could be used to eliminate an unsuccessful medium. Acceptable levels of callus present were grouped together within the marked outline (Table IV.20). and the results presented below were logically based on the media within the outline for detecting the best root promoting medium/media for further refinement.

4.3.4.1 Media series-a (Fig IV.25)

Explants subject to the control treatment (a1, Fig IV.25) exhibited little rooting and as such was significantly less ($P<0.001$) than all other treatments with the exception of treatment media a7 (0.4 mg/l NAA) which was not significantly different. With increasing concentrations of NAA from 0.01 to 0.5 mg/l mean root number decreased from 8.5 to 4.8 roots. The best treatment was a2-medium supplemented with the lowest amount of NAA (0.01 mg/l) followed by a3 (0.05 mg/l) and a4 (0.1 mg/l) media and they were not significantly different from each other (Fig IV.25).

The roots elongated well after 4 month of culture at all the concentrations of NAA tested (Fig.IV.25).

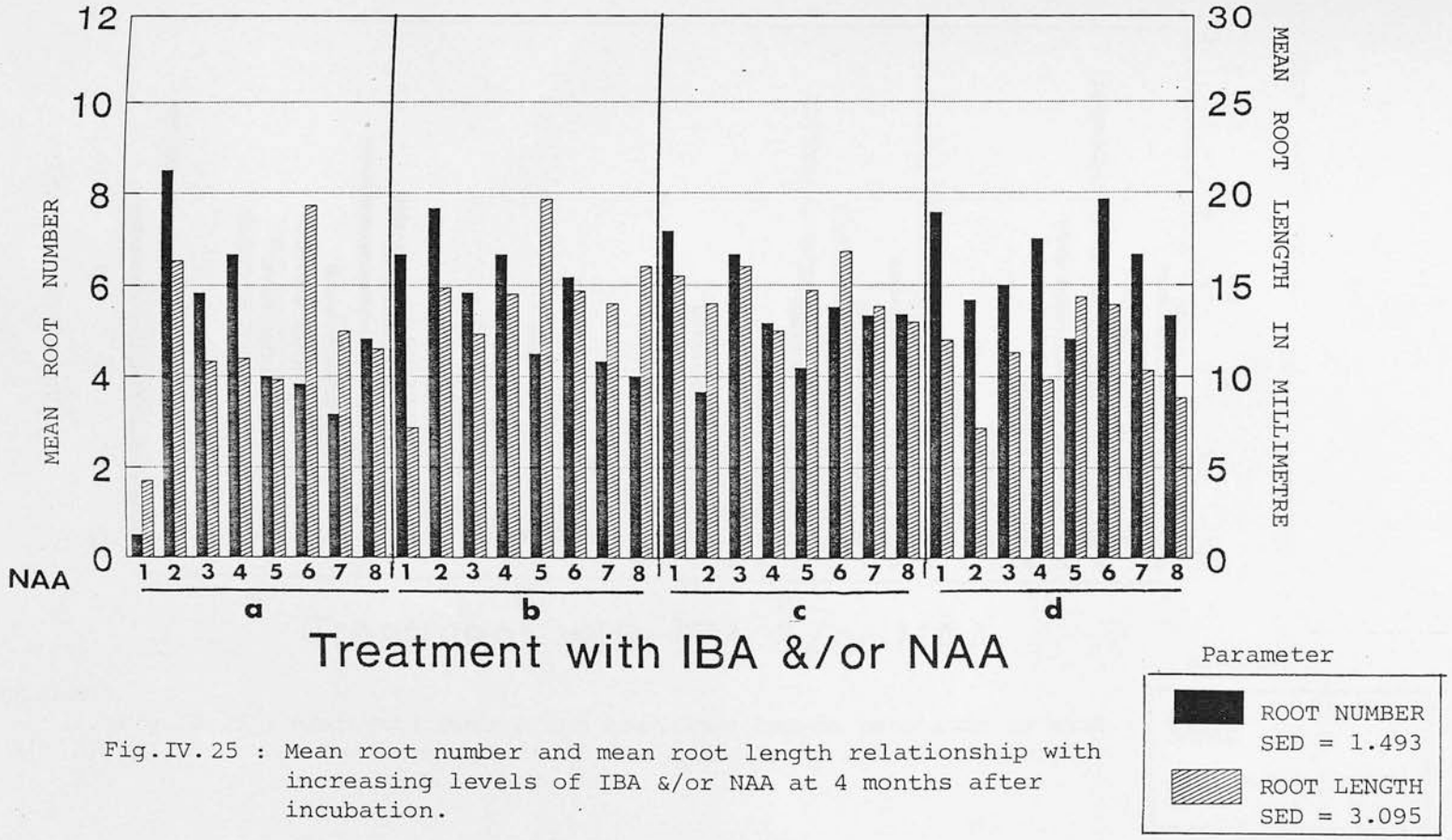
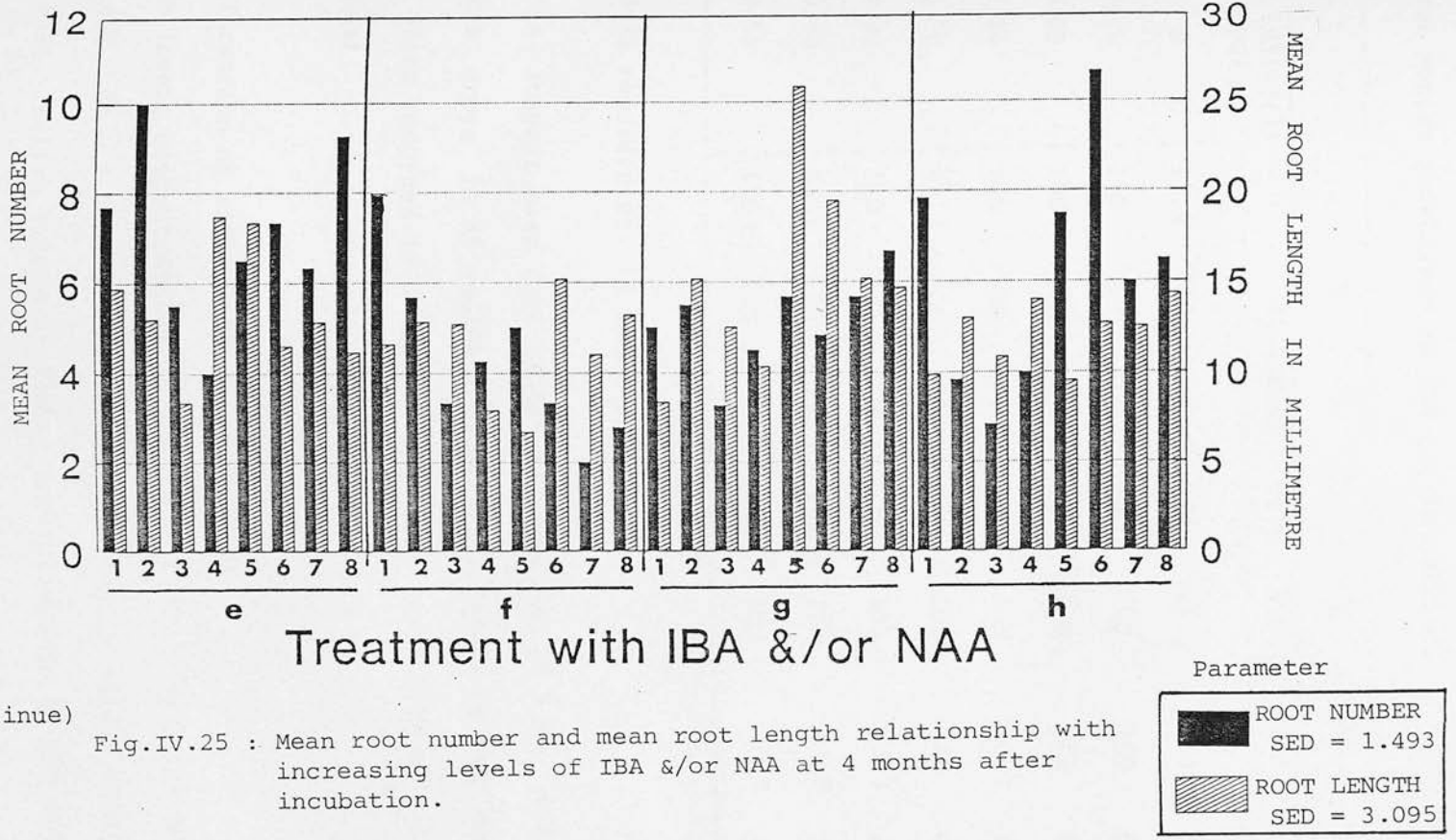


Fig.IV.25 : Mean root number and mean root length relationship with increasing levels of IBA &/or NAA at 4 months after incubation.



(continue)

Fig.IV.25 : Mean root number and mean root length relationship with increasing levels of IBA &/or NAA at 4 months after incubation.

Table IV.20: Rooting percentage of excised shoots (n = 6) with and without growth substances (mixtures of IBA and NAA) treatments *in-vitro* after two months incubation in the growth cabinet.

		a	b	c	d	e	f	g	h
	IBA(mg/l) / NAA(mg/l)	0	0.01	0.05	0.10	0.20	0.30	0.40	0.50
1	0	33.3	100	100	100	66.7	100	100	100
2	0.01	100	100	100	100	100	100	83.3	100
3	0.05	100	100	100	100	100	100	100	100
4	0.10	100	100	100	100	100	66.7	100	100
5	0.20	83.3	100	66.7	100	100	100	100	100
6	0.30	100	100	100	50	100	83.3	100	100
7	0.40	66.7	100	100	100	100	66.7	100	100
8	0.50	100	100	*	100	100	83.3	100	100

* no data recorded due to contamination

The longest mean root length recorded was 19.3 mm, obtained from explants grown in a6 medium (0.3 mg/l) followed by a2 medium (0.01 mg/l) which recorded 16.3 mm. However,both means were not significantly different.

(i) Formation of callus and root branching

At the lowest concentrations of NAA used (0.01, 0.05 mg/l) no callus was recorded. At 0.1 mg/l NAA there was apparent, a slight darkening at the root base. Callus formed at higher concentrations of NAA and peaked at 0.5 mg/l with a callus diameter of 7.8 mm (Table IV.21). In general, induced roots grown on agar medium were thick , unbranched and devoid of

Table IV.21: Average diameter (mm) of callus per explant in the rooting treatment of excised shoots in media supplemented with a mixture of auxins (IBA and NAA) or without after two months of incubation.

		a	b	c	d	e	f	g	h
NAA(mg/l)	IBA(mg/l)	0	0.01	0.05	0.10	0.20	0.30	0.40	0.50
1	0	0	0	0	0	0	0.5	1.0	2.3
2	0.01	0	0.5	3.3	3.5	1.5	5.2	3.5	5.5
3	0.05	0	1.0	3.3	2.5	1.8	3.7	5.2	7.5
4	0.10	0	4.3	5.0	4.0	1.0	4.3	5.3	4.5
5	0.20	1	4.7	6.8	4.0	2.0	0.7	5.7	4.0
6	0.30	0.3	5.5	6.0	5.3	1.0	5.8	1.3	4.3
7	0.40	1.2	5.2	5.2	6.0	4.2	4.3	6.8	6.8
8	0.50	7.8	6.5	*	5.2	5.0	5.5	10.0	8.0

* no data recorded due to contamination.

root hairs. However, one explant grown in medium a5 (0.2 mg/l) produced strong single branched root. Swelling at the root base was recorded on media supplemented with 0.4 and 0.5 mg/l NAA and was a feature common in the entire experiment. The swelling became enlarged after some time in culture,so that at one point the swelling of each root bases were united and became a bigger callus.

4.3.4.2 **Media series-b** (0.01 mg/l IBA), c and d (Fig IV.25)

Explants subject to b2 medium (+ 0.01 mg/l NAA), which had equal amounts of IBA and NAA added to it showed **the best** root production (mean root number (MRN)= 7.7) in this series. This was followed by explants grown in media b1 (0 mg/l NAA; MRN = 6.7),and b3 (+0.05 mg/l NAA; MRN = 5.8) which were not significantly different ($P<0.001$) from each other. Mean root number decreased as the concentration of hormones increased.

The mean root lengths (MRL) recorded was highest in b2 medium (14.8) followed by b3 (12.3mm) and they were not significantly different ($P < 0.001$) from each other. Medium b1 generated the lowest mean root growth (7.2 mm) and was significantly different ($P < 0.001$) from rooting responses shown in b1 and b2.

Series-b medium showed a high degree of callus formation at the root bases (Table IV.21). The higher the auxin concentration in the medium the more likely was callus to form at the root bases and often become jointed together forming into a bigger callus, as found in media b4, b5, b6, b7 and b8, which had a steady increase of callus means diameter from 4.3 mm to 6.5 mm. However, media b1, b2 and b3 had 0, 0.5 and 1 mm mean diameter of callus forming at their root bases respectively.

In the case of media series c (Fig IV.25), c1 which had 0.05 mg/l IBA added to it generated the highest number of roots (7.2) and this was followed by the combination medium c3 (6.7) which had equal concentration of IBA and NAA (0.05 mg/l). Rooting responses of both media above were not significantly different at $P < 0.001$. However, mean

value for the number of roots produced in c2 was the lowest (3.7) and was significantly different ($P < 0.001$) from c1 and c3 above. The lower average value in medium c2 was mainly was mainly due to one replicate which had two roots each, however, the roots are branched and the plantlets produced were of good quality.

Mean root lengths obtained in all the treatments were not significantly different from one another ($P < 0.001$). They exhibited relatively good root growth with a general mean of 15.2 mm.

Mean root number produced in the d-series media d1, d2 and d3 have shown relatively good responses with a mean of 6.2 roots generated per explant. Again IBA alone consistently generated high root production and particular d1 (0.1 mg/l IBA) had a high MRN (7.6). The combination media d2 (MRN = 5.7) and d3 (MRN = 6) had lower MRN than d1 above, however, all three media were not significantly different. Mean root lengths recorded in d1 (12 mm) and d3 (11.3 mm) were lower than c1, c2 and c3 above but they there were not significantly different from each each other. Medium d2 generated the lowest mean growth in length (7.2 mm) and were significantly different ($P < 0.001$) than root growth in c1, c2 and c3 media.

Callus formation in media series-c and d, showed a similar trend to that described for media series-b (Table.IV.21) that is that increasing NAA concentrations in combination with IBA caused a higher degree of callusing of explants at root bases.

4.3.4.3 Media series-e (0.20 mg/l IBA)

Generally, the mean root number produced in the e-series tended to decrease as the NAA concentration was increased up to level e4, after which an increase in the NAA concentration effected an increased in the number of roots produced. Medium e1, which contained 0.2 mg/l IBA alone produced a high number of roots (MRN = 7.7). No callus was recorded. However, the best medium in the series for producing the highest mean number of roots (10) was medium e2 supplemented with a combination 0.2 mg/l IBA and 0.01 mg/l NAA. One other notable medium in this series was e6 (0.3 mg/l NAA) which produced a mean of 7.3 roots per explant tested. Root production obtained from media e1 and e6 were not significantly different from that of e2. Their root growth after 4 months incubation, shown by their mean root length (13 mm), were similar (Fig.IV.25).

Callus formation in the combination media was less in the e-series, with the exception of media e7 and e8 (Table IV.21) which produced a lot of callus at the root base.

4.3.4.4 Media series-f, g and h

In media series-f, f1-medium with 0.3 mg/l of IBA added to it again exhibited similar root promotive effect as in media b1, c1, d1 and e1 (Fig. IV.25) . A mean root number (8) was stimulated, however, some callusing (0.5 mm diameter) was also evident . In the combination experiments, only f5-medium showed very little callusing (Table IV.20), its mean root number (5) and mean root length (6.7 mm) values were comparatively low.

Media g1 which had 0.40 mg/l of IBA added to it produced a mean of 5 roots per explant and a corresponding mean root length of 8.3 mm

and these were the lowest values recorded in the IBA-media series. In the media g2 (with 0.01 mg/l NAA) and g6 (with 0.30 mg/l NAA), the mean root number recorded was 5.5 and 4.8 roots with a mean root length of 15.2 and 19.5 mm respectively.

Medium-h1 (0.50 mg/l of IBA alone) produced a high MRN (7.8) and a low mean root length (9.8 mm).

All combination media in the f-series (exception was f5), g-series (exception were g2 and g6) and h-series, generally showed a high degree of callus, as much as 10 mm diameter of callus was found in medium g8 (0.4 mg/l IBA + 0.05 mg/l NAA).

4.3.5 Discussion

This study has shown that rowan shoot explants obtained from the shoot proliferation experiments were able to root *in vitro* by employing two auxins (IBA and NAA), either supplemented singly or in combinations in half-strength MS media, low in agar and sucrose content, 0.6 % and 0.8 % respectively.

It is interesting to note that for the entire experiment, there were many media capable of inducing rooting of excised shoots and nodal segments. In this experiment the explants used were a mixture of nodal segments and shoot tips, however, generally nodal segments were most used due to their availabilty from the previous shoot proliferation experiments. The best media that produced good rooting, but also had low callus formation were a2 (0.01 mg/l NAA), a3 (0.05 mg/l NAA), a4 (0.10 mg/l NAA), b1 (0.01 mg/l IBA), c1 (0.05 mg/l IBA), d1 (0.10 mg/l IBA) and e1 (0.20 mg/l IBA).

In earlier experiments (section IV.4.3.2) with a high sucrose level, NAA, induced more cases of root base swelling which eventually led to callus formation over time. This is similar to the present experiment using hormone combinations, callus tended to increase as concentrations increased. It would be interesting to see the effect of separating the root initiation stage and the root elongation stage. Once root initials are obtained, the explants could be transferred to a basic MS medium. Unfortunately, this was not done due to shortage of explant materials.

4.4 Experiments on hardening off of *in vitro* rooted shoots

4.4.1 The influence of auxins and a hormone rooting powder in rooting formation of excised shoots *in vivo* (Experiment 1)

The presence of different types of PGRs and their concentrations markedly influenced the rootability and rooting performance of excised shoots (Table IV.22 and Fig IV.26a). The presence of an auxin is essential for the induction of adventitious roots. The application of the commercial rooting powder, Seradix 3, was the best ($P \leq 0.01$) to promote the rooting of excised shoots *in vivo*. An analysis of variance revealed a significant difference $P \leq 0.01$ between treatments. Seradix 3 induced the highest rooting percentage, (56.7 %) (Table IV.22). Excised shoots treated with IBA at lower concentrations, 100 mg/l or with a higher concentration of NAA, 500 and 1000 mg/l or without any hormone treatment (control) produced the lowest rooting percentage (Table IV.22 and Fig IV.26a).

The mean root number and mean root length obtained (Fig IV.27) showed excised shoots treated with Seradix 3, was significantly ($P \leq 0.01$) different from all other treatments producing the highest values

Table IV.22: Rooting percentage of excised shoots in vivo (n = 30) with and without hormone treatment in peat and sand mixtures after 2 months in a controlled environment.

Treatment	% rooted	% unrooted	% mortality	MRN	MRL
Control	3.3	0	96.7	0.03	0.13
Seradix	56.7	26.7	16.7	2.60	26.10
100 mg/l IBA	3.3	23.3	73.3	0.20	0.57
250 mg/l IBA	26.7	46.7	26.7	0.53	2.63
500 mg/l IBA	13.3	40.0	43.3	0.40	0.60
1000 mg/l IBA	30.0	36.7	33.3	1.20	1.61
100 mg/l NAA	23.3	40.0	36.7	0.43	3.17
250 mg/l NAA	13.3	23.3	63.3	0.13	1.20
500 mg/l NAA	0	16.7	83.3	0	0
1000 mg/l NAA	10	6.7	83.3	0.20	0.53
SED	9.67	15.23	18.22	0.2731	1.272
DF	9	9	9	9	9

of 2.6 and 26.1 mm respectively after two months of planting.

Rooted plantlets induced by Seradix (Plate IV.11), IBA (Plate IV.12a) and NAA (Plate IV.12b) produced good quality fibrous roots, however, NAA treated roots tended to be smaller in size.

Percentage mortality of excised shoots were highest in the control (96.7 %) followed by 500 and 1000 mg/l levels (both showed 83.3 % mortality), IBA treated shoots at 100 mg/l (73.3 %) and NAA treated shoots at 250 mg/l (63.3 %) (Fig. IV.26c). Lowest percentage mortality was recorded for the Seradix treatment (16.7 %).

Plate IV.11 : Rooted plantlets induced by Seradix

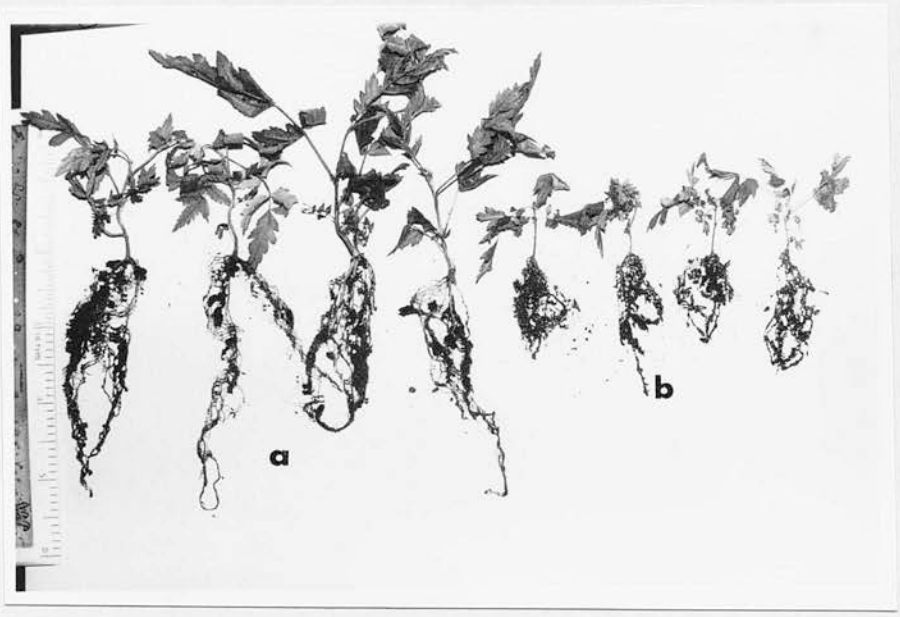


Plate IV.12: Rooted plantlets induced by (a) IBA and (b) NAA

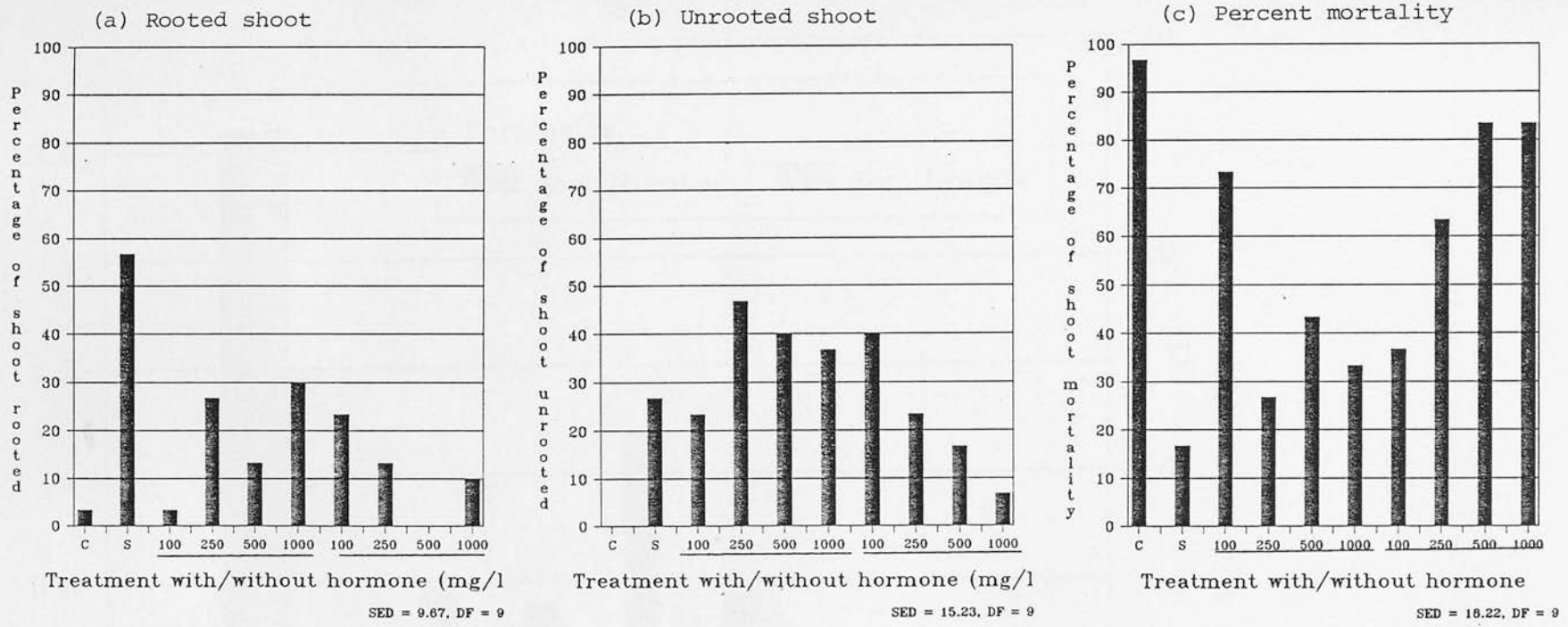
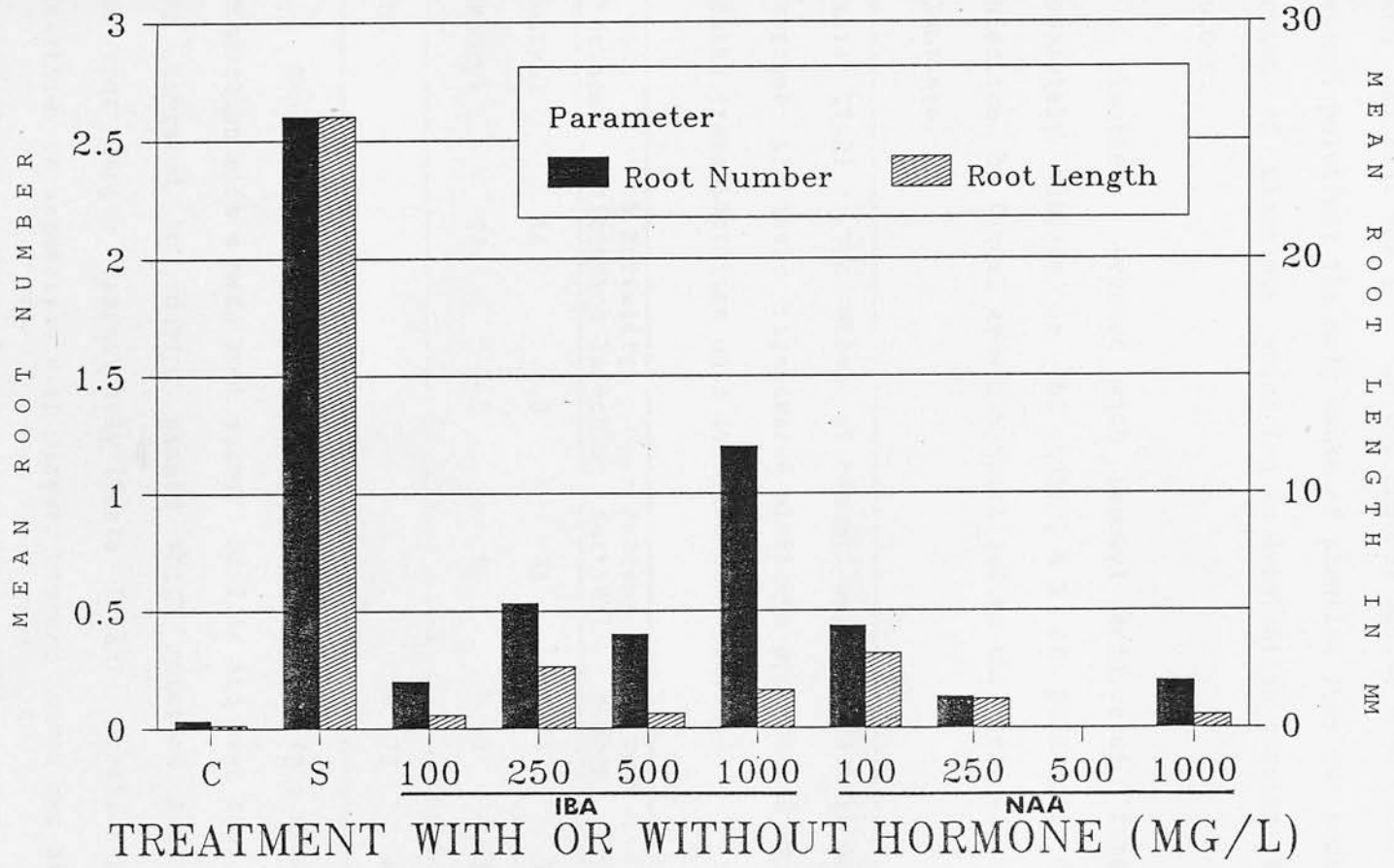


Fig.IV.26 : Percentage of shoot (a) rooted, (b) unrooted and (c) mortality when treated with Seradix (S), IBA and NAA. The Control (C) sterile water.



SED = 0.2731 (MEAN ROOT NO.)
SED = 1.2720 (MEAN ROOT LENGTH)
Df = 30

Fig. IV.27: Mean root number and Mean root length relationship when treated with Seradix, IBA and NAA. The Control (C) - washing with sterile water.

4.4.2 Experiment on the hardening off *in vitro* rooted shoots for transfer to soil in the greenhouse (Experiment 2)

As illustrated in Table IV.23 percentage survival of the newly regenerated plantlets after transplantation onto the rooting medium of sand and peat was 48 % in the control and 56 % in those treated with benomyl solution. The main cause of plantlet loss was possibly excessive drying of plantlets which led to dessication turning them brown in colour.

Plantlets treated with benomyl eradicated fungal infection completely whereas in the control 8 % of plantlets developed root infection by fungal mycelium which led to the eventual death of these plantlets.

Table IV.23 The effect of benomyl solution (0.5 %) on root growth response of newly regenerated plantlets produced *in vitro* after 2 months transplantation onto peat and sand mixtures.

Root Treatment	% Mortality desiccation	Infection	Percent survival	Mean root number	Mean root length(mm)
Control	44	8	48	1.64	14.2
Benomyl	44	0	56	2.68	18.3
SED				0.279	1.46

Benomyl-treated plantlets had significantly ($p \leq 0.01$) better root production with a mean root number of 2.68 and mean root length of 18.3 mm compared to control plants which recorded 1.64 roots and 14.2 mm root length respectively (Table IV.23). Treated plantlets were healthier in appearance with bigger, greener leaves and bigger stem size (Plate IV.13), although the adventitious roots formed, generally shows

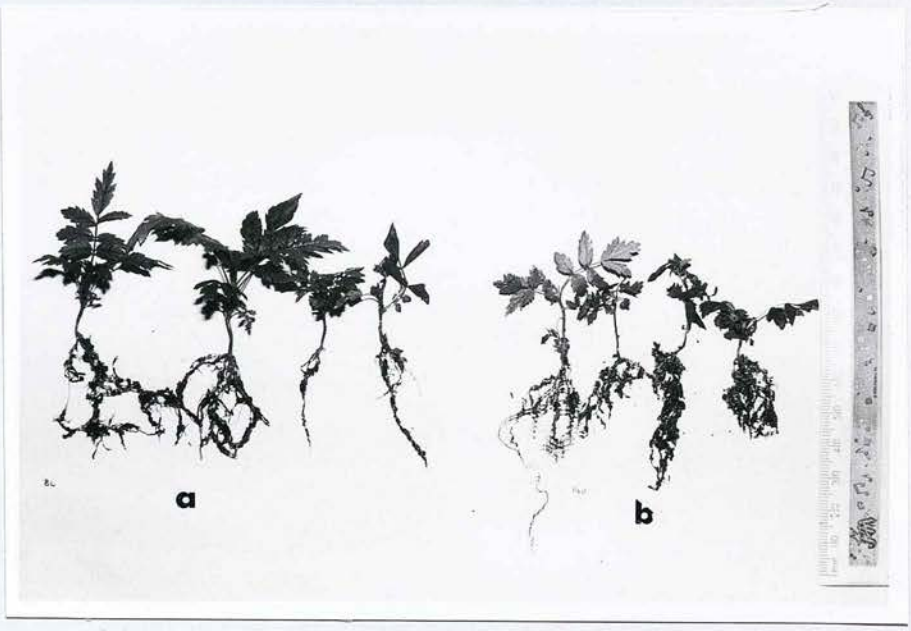


Plate IV.13 : The effect of (a) benomyl solutions (0.5 %) on general growth response of newly regenerated plantlets produced in-vitro after 2 months transplanting onto peat and sand mixtures and (b) washing them with sterile water (control) and transplanted as in (a)

similarity in appearance in both treated and untreated (Plate IV.13). Both groups of plantlets developed a root system which was fibrous and of good quality.

4.4.2.1 Discussion

Acclimitization of newly regenerated plantlets produced *in vitro* into soil is critical to any micropropagation programme. In the above experiment,. it was evident that the plantlets was very susceptible to drying out. The high mortality rate due to dessication was more evident for those plantlets located around the edges of the germinators, where continous air currents caused by the growth cabinet fans enhanced the desiccation process.

Washing the roots of *in vitro* produced plantlets with sterile water or benomyl solution increased the percentage of plants successfully acclimitizing by reducing loss due to fungal infection. However, it was not clear whether increased rooting of plants treated with fungicide was entirely due to the control of diseases, or a synergistic and/or hormonal effect on roots by the fungicide. In other studies by Grigsby(1965), a fungicide, Captan, was reported to significantly increase the rooting of cuttings, but the increase in rooting was related to the fungicide concentration used. Captan also increased the percent survival of treated cuttings (Grigsby, 1965; Hansen and Hartmann, 1967).

4.4.3 Experiment on *in vivo* rooting of shoots in the greenhouse condition (Experiment 3)

Thirty out of fifty excised shoots that were treated with Seradix 3 survived forming normal and healthy plantlets. After four months from the first planting (Plate IV.14), the micropagated plantlets grew into

Plate IV.14 : Four months old plantlets of Sorbus aucuparia L originated from shoots treated with Seradix 3.

normal, healthy plants acclimitized to outside conditions. The seedlings showed no apparent abnormalities .

4.4.4 Discussion of experiments on hardening off rooted shoots (Experiments 1 - 3)

In this investigation, excised shoots of *Sorbus aucuparia* L. that arose from cultured nodal explants rooted easily *in vivo* , but different types of PGR's and their concentration influenced rooting percentage. Higher concentrations of IBA (1000 mg/l) were effective for root formation whereas NAA were more effective at lower concentrations (100 mg/l, Fig.IV.26). Seradix 3, a commercial rooting PGR was the best among the three PGRs tested. The success and survival of rooted plantlets by Seradix treatment may be attributed to several factors. Firstly, its composition of 0.8 % IBA with a fungicide added to it, stimulated rooting as well as providing protection of the cut surface of the shoots from fungal infection. Secondly, Seradix powder tends to adhere longer to the cut surface and this makes it more effective at providing a longer lasting effect to encourage root initiation and formation.

The success of transplantation and survival of plants greatly depended on the quality of roots formed. In the present study, all successfully rooted plantlets produced good quality roots regardless of the PGR used, however, they differed in their fibrosity , number and overall size of the root mass. IBA- and Seradix induced roots produced better quality roots than those of NAA-induced ones. A similar result was reported for pear (*Pyrus communis*) plants (Lane, 1979).

Heavy losses of the excised shoots recorded was mainly due to dessication. Water loss from the excised shoots can happen at various stages of *in vivo* propagation. *In vitro* produced shoots are much smaller compared to those produced by conventional cuttings, and therefore are more susceptible to dessication during auxin treatment and insertion into the rooting medium. In addition, the plastic covers were loosely placed on top of the explants trays which was thought to be desirable for good plant growth, by providing limited aeration into the germinator, however, this provided an airflow which greatly reduced the humidity in the germinators and quickly dried up the plantlets. Such explants are particularly sensitive to dessication as has been reported by Zimmermann and Broome (1980), because leaves produced *in-vitro* have an incomplete and poorly developed cuticles. Fuchigami *et al.* (1981), also concluded that water loss occurred mainly on the abaxial surface of leaves where most stomata are located. A significant cause of water loss is due to a slow stomatal response (Nemeth, 1986).

This study is the first to report *in vivo* rooting of micropagated shoots in *Sorbus aucuparia* L. and the technique used has many benefits, provided improvements are made to the control of humidity, by using for example a mist propagator or by spraying the plants with an antitranspirant. In addition, fertilizers may improve the establishment in the soil (Lane, 1979; Snir, 1983; Cheema and Sharma, 1983). The cost of production could be reduced by switching the rooting stage from an *in vitro* step to an *ex vitro* one. This would mean that the shoots for rooting could be handled as miniature cuttings (microcuttings) without using aseptic techniques. This change permitted the combination of rooting stage with acclimitization stage which would cut costs and time.

Using the commercial rooting powder like Seradix is not only cheap but allows the whole procedure of rooting and acclimitisation to be performed quickly, thus more shoots could be rooted with it being simpler and faster to transplant rooted shoots into pots. No callus was recorded using this technique and this was certainly more desirable.

The technique of treating the cut basal ends of micropropagated shoots with an auxin carried in talc powder then inserting them into a rooting medium or peat plug, which were then placed under mist or high humidity conditions worked successfully with blackberry, *Rubus spp.* (Broome and Zimmerman, 1978; Zimmerman and Broome, 1980b), blueberry, *Vaccinium elliottii* (Zimmerman and Broome, 1980b) and apple, *Malus spp.* (Zimmerman and Broome, 1980c; Simmonds, 1983).

SECTION V

GENERAL DISCUSSIONS AND CONCLUSIONS

1. PRELIMINARY SURVEY OF SEED PRODUCTION OF THREE BROADLEAVED TREES

The study on seed production demonstrated that young trees of reproductive age produced less fruits but of good quality, whereas an overmature tree fruited enormously but produced seed of poor quality.

Observations on the flowering behaviour of rowan trees, an understorey species, grown in a mixed woodland with a semi-open canopy provided evidence of an unexpectedly low seed production (7%) which is contrary to common observation of trees grown as ornamentals in the open. Factors involved in the production of flowers, fruits and seeds are many. One of the crucial factors to maintaining adequate supplies of good seeds is a sound knowledge of seed production (Bonner, 1988). A more detailed study spanning several flowering seasons is needed to further knowledge on flowering and fruiting behaviour of rowan.

2. SEED DORMANCY IN ROWAN (*SORBUS AUCUPARIA* L.)

The main objective of the first part of the study was to investigate further the nature of the dormancy and its after ripening and to confirm some earlier findings in the seeds of rowan.

2.1 After-ripening

2.1.1 Effects of moist-cold stratification

The moist-cold stratification studies of rowan seeds generally confirmed earlier findings (Flemion,1931; Hilton *et.al.* 1965; Zentch, 1970 and

Lenartowicz,1988). Results obtained by Flemion (1931) were similar in that approximately 16 weeks (4 months) were needed to break dormancy. Stratification at 1°C was superior to that at 4°C, commencing an earlier IGP which was of shorter duration (Figs. IV.1a, Table IV.3). Despite this the 4°C temperature was chosen for subsequent experiments due to its easier maintenance.

There were large variations in the requirement of chilling periods to break dormancy of all seeds in the same seedlot, which was evident from the duration of IGP at 1°C and 4°C stratification temperatures (Figs.IV.1a, b, c and d). The duration of IGPs of all the stratification experiments varied considerably from 5 to 16 weeks. This reflected the wide range in the depth of dormancy of seeds obtained from British Seed Houses Ltd. However, freshly collected seeds from a single tree had the shortest IGP duration (5 weeks) and more importantly achieved a very high percentage of germination. It is reasonable to suggest that for rowan seeds a more synchronous (hence a shorter IGP) and a better germination performance could probably be obtained by stratifying fresh seeds at 1°C.

2.1.2 Effects of alternating temperature treatment

A large number of plant species which grow in temperate regions have seeds which require alternating temperatures for dormancy breaking. In nature the response is often difficult to quantify since many variables are involved, including temperature, magnitude of fluctuation, the length of exposure to each temperature and the number of cycles (Bryant, 1985). However, this can be simulated in the laboratory by controlling the time and temperature of the warm and cold periods.

Two weeks warm moist stratification at 20°C, following 22 weeks at 4°C improved the total germination from 63 % (Fig. IV.1d) to 98 % (Fig. IV.3). This confirmed reports made by Gordon and Rowe (1982) and Lenartowicz (1988). However, the IGP duration was lengthened from 5 to 7.5 weeks (see Table IV.3, Fig IV.1b and Fig IV.3). These studies indicate that the duration of IGP is also an important criterion for evaluating the effectiveness of seed treatment. This agrees with Lenartowicz, (1988). Thus, 2 weeks at 20°C followed by a cold (4°C) stratification was not sufficient to induce maximum germination. A longer warm-phase of 6 months at either 10°C, 15°C or 20°C followed by a cold phase at 4°C of variable length; 11 to 20 weeks (see Figs. IV2a, 2b and 2c), however, shortened the duration of IGP from 7 - 16 weeks (Figs. IV.2a, c and d) to between 3 - 5.5 weeks. The best temperature, that accelerated IGP to 3-weeks and ended it in the shortest time (11 weeks) and produced a high germination percentage (84%), was 20°C. Lenartowicz (1988), had found that the most synchronized germination of a population of *S. aucuparia* seed could be obtained by stratification at 1°C or 3°C preceded by 10 weeks (2.5 months) at 15° - 25°C producing 80% germination.

2.1.3 Effects of stratification medium

The study on stratification media demonstrated that a substrate can modify dormancy breaking behaviour in cold stratified seeds. Flemion (1931) found that peat was the best medium compared to sand which she attributed to its low acidity [pH ± 4] which had the effect of weakening the seedcoats, better than sand. However, in my study the highest percentage of germination after 10 weeks of stratification at 4°C was

obtained in the order of sand > vermiculite > peat > polythene bag. The medium of stratification also influenced the post-dormancy growth of seedling. The optimum for root growth was obtained from seeds that after-ripened in sand whereas for shoot growth and leaf production peat was the best medium. Following this, it is reasonable to suggest that the best stratification medium would be a mixture of sand and peat. For lengthy moist-stratification of seeds of rowan, the use of polythene bags are not recommended unless proper aeration and frequent changes of the bag are conducted to reduce the accumulation of toxic metabolic by-products which affected seedling health, although TG was found to be high. For research work, stratification in polythene bag afforded the convenience that stratified seeds could be cleaned more easily compared to peat, sand or vermiculite. This is helpful when aseptic conditions are adopted.

2.1.4 Effect of PGR on dormant embryos

The major finding of the present study was that exogenous application of low concentrations of the cytokinin BAP, could closely reproduce the effect of chilling in dehulled seeds. This disputes Nikolaeva's (1977) finding that no treatment can successfully break dormancy of deeply dormant seeds (see Table I.1) which normally needs protracted prechilling to after-ripen. The effective concentration of BAP to after-ripen dormant rowan embryos, was considerably less (0.1 mg/l) if incorporated in agar media with half-strength MS solution whereas a higher concentration (2 mg/l) was needed if applied to paper substrate in petri-dishes. Both conditions were the best to promote root, hypocotyl and shoot growth resembling that of normal after-ripened seeds. Hence, for rowan embryos, dormancy results from insufficiency of

BAP synthesis or an inability to release bound CK, K and 6-benzylaminopurine (BAP), however, was reported to have little effect on germination by themselves but acted synergistically with light (Miller, 1958). In my study, exogenous applications of the BAP were all conducted at 20°C, in continuous light. It would be interesting to see the effect of BAP on dormant embryos in the dark. It is also known that CK is involved in the process of radicle elongation (Haber and Luippold, 1960; Pinfield and Stobbart, 1972) and cotyledon expansion (Kursanov *et.al.*,1969; Ikuma and Thiman, 1963; Hutton *et.al,* 1982) and that they are transported between the cotyledon and embryonic axis (Grepstein and Ilan, 1979). In my study, GA_3 treatment had no effect on embryo development. Auxin treatment was generally ineffective in inducing growth in embryos, although NAA treatment promoted root growth.

Few results relate change in CK to seed dormancy. In several cases, the CK level increased with time suggesting a functional role in after-ripening (Kopecky, Sebanek and Balzkova, 1975; Webb, Van Staden and Wareing, 1973a). A decrease in CK during stratification is also reported (Webb, Van Staden and Wareing, 1973b). Transient increase in various GA's and CK's takes place in seed of some species during both the breaking of dormancy and the process of germination (Van Staden et.al., 1972; Brown and Van Staden, 1973; Borkowska and Rudnicki, 1975; Taylor and Wareing, 1979; Julin-Tegelmen and Pinfield, 1982), but the timing of the events does not seem to be related in any clear pattern to the course of dormancy breaking (Bewley and Black, 1986). The latter observation may stem from some shortcomings of the above studies wherein very few of the CK's were unequivocally identified (Bradbeer, 1988). In

addition, varying bioassays were employed and thus lack standardisation and also the exact function(s) of the different CK derivatives which were present in seeds and fruits were not known.

In this study CK's in testa, mesocarp and embryos were extracted after various stratification periods using TLC techniques. At least 4 CK's were identified and are the first reported in this species. Based on a comparison of standards and Rf values obtained from the literature their identity could be dihydrozeatin, a purine, cis-zeatin and one unknown. In the embryo, dihydrozeatin appeared to decrease during stratification. A BAP-like CK (purine) was present in testa and mesocarp after 6 and 8 weeks of stratification. It is difficult to interpret the results of the CK extraction experiment. However, the results obtained by Hutton and Van Staden (1982a ; 1982b) showed that labelled zeatin applied to germinating seeds were implicated in the formation of 6-(2-3-4-trihydroxy-3 methyl butyl amino) purine which ultimately brings about side chain cleavage, apparently by oxidation, and resulting in N-(puryl-6-yl)glycine as an end product. This step may well play an important role in regulating CK levels within plant tissues and pinpoint the exact function of the CK's in the germination process (Van Staden, 1983). The decreased level of dihydrozeatin in the embryos after stratification (section IV.3.9.2) may infer that it may be implicated in the formation of the purine which produced the effect of BAP in the release of dormancy in dormant rowan embryos. The presence of dihydrozeatin, in substantial concentration, in all the seed parts throughout the stratification period may imply its important function, such as 'dormancy breaking', and that its reduction was perhaps activated by the chilling temperature.

No attempt was made in this study to identify inhibitors that were reported in rowan by Barton and Solt (1949) and Hilton *et.al.*, (1965). Interpretation of such data by the activator-inhibitor hypothesis (Khan, 1975) is often difficult (Taylorson and Hendricks, 1977). It is suspected that ABA, a water-soluble inhibitor is present in dormant rowan seed. If ABA is implicated, its effect could be nullified by CK (Robert and Hooley, 1988). In addition, CK is reported to have the ability to reverse inhibition of germination caused by naturally occuring inhibitor.

For further study, it is felt that a more intensive sampling of seed parts from cold stratified rowan seeds over 14 to 15 weeks and the CK extracts run on HPLC, would clarify further the role of CK in the dormancy of rowan. In addition, for understanding more precisely the mechanism of action of the growth regulators in breaking dormancy, it is worth examining simultaneously the nature and level of promoters and inhibitors in seeds.

In conclusion, the rowan embryo dormancy resulted from an insufficiency of cytokinin-like synthesis or an inability to release bound CK.

2.1.5 The role of seed coverings

The fact that intact seeds germinated much more slowly than the embryos from stratified seeds when germinated in petri-dishes,especially at the early stratification periods (4, 6 and 8 weeks), demonstrated that the seedcoat plays an important role in the dormancy of rowan. Furthermore,

Flemion (1931) found that rowan embryos after-ripened in six weeks in contrast to intact seed which needed 8 - 16 weeks of cold stratification. The embryo coverings which are composed of the testa and the mesocarp are tough seed coverings that provide mechanical restraint to embryo growth. It appeared that after-ripening of the embryo was complete before the seedcoats are deteriorated enough to allow germination. Therefore rowan seed is also subject to physical dormancy and this was artificially broken by incision of the appropriate size which resulted in germination by exogenous application of 2 mg/l BAP. This physical restriction perhaps can be overcome by natural events, such as moist cold conditions or scarification by partial digestion through the gut systems of birds (see Section I, 2.2.1.4 (v). In addition, the activities of microorganisms in nature help to weaken the tough coverings of rowan seed thereby effecting an earlier release from its physical dormancy or perhaps by natural selection, synchronising it to the onset of its embryo release from dormancy. The effects of fungal and viral infection on the seed has been mentioned (section IV.3.3). Some seeds were resistant to microbial infection and if affected, they degraded the seedcoats much quicker thereby releasing its mechanical resistance to embryo growth. Unsterilised seeds , not only began and ended their IGP period much faster than sterilised ones, but also shortened its duration by a month when compared to the sterilised seeds.

2.2 Tests of viability

2.2.1 Excised-embryo test, tetrazolium test and greening of cotyledons

A quick test of viability for rowan seeds was performed quite easily by the excised-embryo test and the mean value obtained for seeds from British Seed Houses Ltd. was around 73%. If comparison is based on TG =

66 % obtained from 4°C stratification (Fig IV.3.1c) the above estimate was good. But if it was based on the actual potential of seed germinated by the long warm-phase alternated with the cold phase (Fig. IV.2a, 2b and 2c) the value obtained for EET was underestimated. It was even more underestimated by the TTZ test with a value of around 62%. As reported in Kozlowski (1972), Shubert [1965] compared the 'excised-embryo test' with the TTZ test method in a comprehensive study for determining the viability of dormant tree seeds. He concluded that the TTZ method should receive preferance over the EET. However, TTZ test requires skilled personnel to produce reliable results and knowledge on staining behaviour of the seeds for a correct classification of viable seeds.

This study has demonstrated the cotyledon greening behaviour of the embryo both with and without plant growth regulator treatment [PGR]. Depth of dormancy can be assessed by the percentage of greening of the embryos when placed in GT conditions. A fully green cotyledon also reflects seed vigour and shallower dormancy. Semi-greening will reflect deeper dormancy depending on the percentage of greening. At the other extreme when the cotyledons remain unchanged (white or creamy) but fresh, it is said to have the deepest dormancy. The observation that the application of exogenous BAP improved cotyledon greening of the embryos showed that the level of CK was inadequate for full greening. However, this improvement of cotyledon greening and later after-ripening of the embryo occured in the presence of light.

3. MICROPROPAGATION OF *SORBUS AUCUPARIA* L.

The potential use of tissue culture in forest improvement programmes has

been reported by a number of researchers such as, Bonga (1977), Durzan (1982), Rao and Lee (1982), Bonga and Durzan (1982) and Bajaj (1986). However, the application of tissue culture in forestry is still in its infancy although economic large scale micropropagation may not be far away (Bonga and Durzan,1982). Effort must be geared to realize the full potential of these fast developing technologies. Initially improvement will result from the micropropagation of superior genotypes. It is neccessary to produce plantlets from trees mature enough to manifest their genetic worth. Later, however, tissue culture may provide the essential route back to whole plants from germplasm, selected or further improved by new biotechnologies (Timmis *et.al.* 1987). However, the use of embryonic and seedling tissues as a starting point seems to be useful to build the base line information required for developing and refining more reliable procedure for *in vitro* culture of tree species. From earlier work (Mott, 1981; Vieitez *et. al*, 1978) it is clear that the conditions necessary to promote organogenesis in cultured tissue from woody plants must be developed for each individual species.

Besides propagation by seeds rowan can also be propagated by various techniques of bud grafting (Browse, 1988). However, it will not grow from cuttings and layering was reported to be difficult with poor results (Lawyer, 1968). A micropropagation technique is another alternative for propagation of rowan trees and it is probably commercially viable since it is an important ornamental tree with wide distribution throughout the temperate countries.

Micropropagation of rowan plantlets from embryonic and seedling explants in the present study was an attempt to elucidate possibilities

of generating these species *via* tissue culture techniques. This effort was encouraged and initiated from the earlier success in dormancy breaking of embryos mediated by *in vitro* application of the CK BAP.

3.1 Multiple shoot proliferation

The data presented in the second part of the thesis demonstrated clearly that multiple shoot formation was possible from dormant excised embryos. By screening a wide spectrum of BAP and IBA concentrations in various combinations incorporated into a basic MS medium (half-strength) in 0.6 % agar with 3% sugar added with a pH at 5.8; it was possible to identify the optimal combination of PGRs for multiple shoot formation. It was mentioned that a reduction in sugar content in the proliferation medium in the initial experiment produced better shoot production. The best treatment combination for the proliferation of multiple shoots was obtained from 0.2 mg/l BAP with either 0.05 or 0.08 mg/l IBA. The highest average number of healthy shoots obtained was 3.5 shoots (in 6 weeks). These shoots which were maintained in a Gallenkamp incubator at 20°C with a 16h photoperiod for eight months could be excised into nodal segments and shoot tips and could be recultured in the same proliferation medium. The best treatment combination for multiple shoot production was obtained from 0.2 mg/l BAP with either 0.01 or 0.02 mg/l IBA which produced a better shoot growth when compared to those raised from embryos. The average number of 5 shoots per explant (in 6 weeks) was obtained. Better results were obtained with two-months old shoots. The best medium for shoot proliferation in this case was obtained from the medium supplemented with a low concentration of IBA (0.2 mg/l BAP/0.01 mg/l IBA) producing an average of 6 shoots per

explant, the shoots obtained were morphologically superior and healthy in appearance. Chalupa (1981), who used nodal segments from 2 year old actively growing seedlings, found that a slightly higher concentration of BAP and IBA (respectively 0.6 and 0.05 mg/l) in full strength MS medium stimulated rapid growth of multiple shoot from the axillary buds. This result confirms that BAP stimulates production of shoots from axillary buds and suggests that BAP is the most useful and reliable (also the cheapest) CK available. The shoot clusters developing from cultured existing meristems can be divided into smaller groups of shoots and transferred into fresh medium or explants can be excised from developed shoots and subcultured onto fresh medium for further shoot production. It was concluded that axillary nodes of rowan seedling can be substantially enhanced by growing shoots in a medium containing a suitable CK at an appropriate concentration with or without auxin.

It is important to note that beside BAP and IBA and the correct pH of the medium use, another factor that promotes multiple shoot formation is the supply of carbohydrate. Sucrose has been the carbohydrate of choice and the best in the vast majority of reports on shoot induction in woody plant species (Thompson and Thorpe, 1987) and the final concentration between 2 - 4 % was normally used. Further refinement on the optimum concentration, as well as the medium could be tested. In this study half-strength MS was used, as this was recommended for the initial establishment of woody species in new-to culture conditions. The reason for this, is that unlike the high salt media, the low salt media seldom are so inhibiting as to be lethal although they may not be optimal (McCown and Sellmar, 1987).

In conlusion a higher CK to auxin ratio promoted greater shoot proliferation in rowan.

3.2 Rooting of *in vitro* produced shoots

Following the success of the shoot proliferation experiment, shoots generated could then be excised and rooted in a rooting medium. For optimal root formation various rooting media were screened as conducted for shoot multiplication. The excised shoots were regarded as microcuttings. A cutting is a plant suddenly lacking one of its physical and physiological support system, the roots (Gaspar and Goumans (1987). According to Hassig (1985) profound perturbation of normal metabolism is required in order to sustain life in the cuttings. He added, that adventitious rooting may be under genetic control. One reason put forward for this, was the consistent differences found between and within genera in their rooting ability. Plants under water stress conditions either at the base of cuttings or as water loss from leaves to the surrounding air, induce the formation of adventitous rooting (Gaspar and Goumans, 1987). Rooting of excised shoots could be achieved either by subculturing with a medium lacking of CK with or without auxin, or by treating the excised shoots as conventional cutting (Hussey, 1986). The data presented on rooting of excised shoots of rowan in this study were in agreement with the above statement.

It is concluded that the rooting performance (described in section IV. 4.3) showed that auxin, IBA and a low NAA and a lower concentration of sucrose (0.8%) gave better rooting performance, compared to that obtained in a higher sucrose content media. However,

in vitro produced shoots of *S. aucuparia* were much easier rooted *in vivo* in the greenhouse producing a higher rooting percentage by using a commercial rooting powder,Seradix 3, which could be obtained cheaply. It has several advantages, besides aseptic techniques not being required , shoots can be rooted in larger numbers at the same time in propagator boxes or mist chambers. It is also much easier to transplant the rooted shoots into pots.

In conclusion, excised shoots of *S. aucuparia* obtained from cultured nodal explants rooted easily and successfully in a non sterile 50 % peat and 50 % sand mixture in greenhouse conditions as long as enough moisture was provided to keep dessication injury at a minimum. Cuttings needed at least two weeks in a covered propagator before the cover be gradually be lifted off.

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